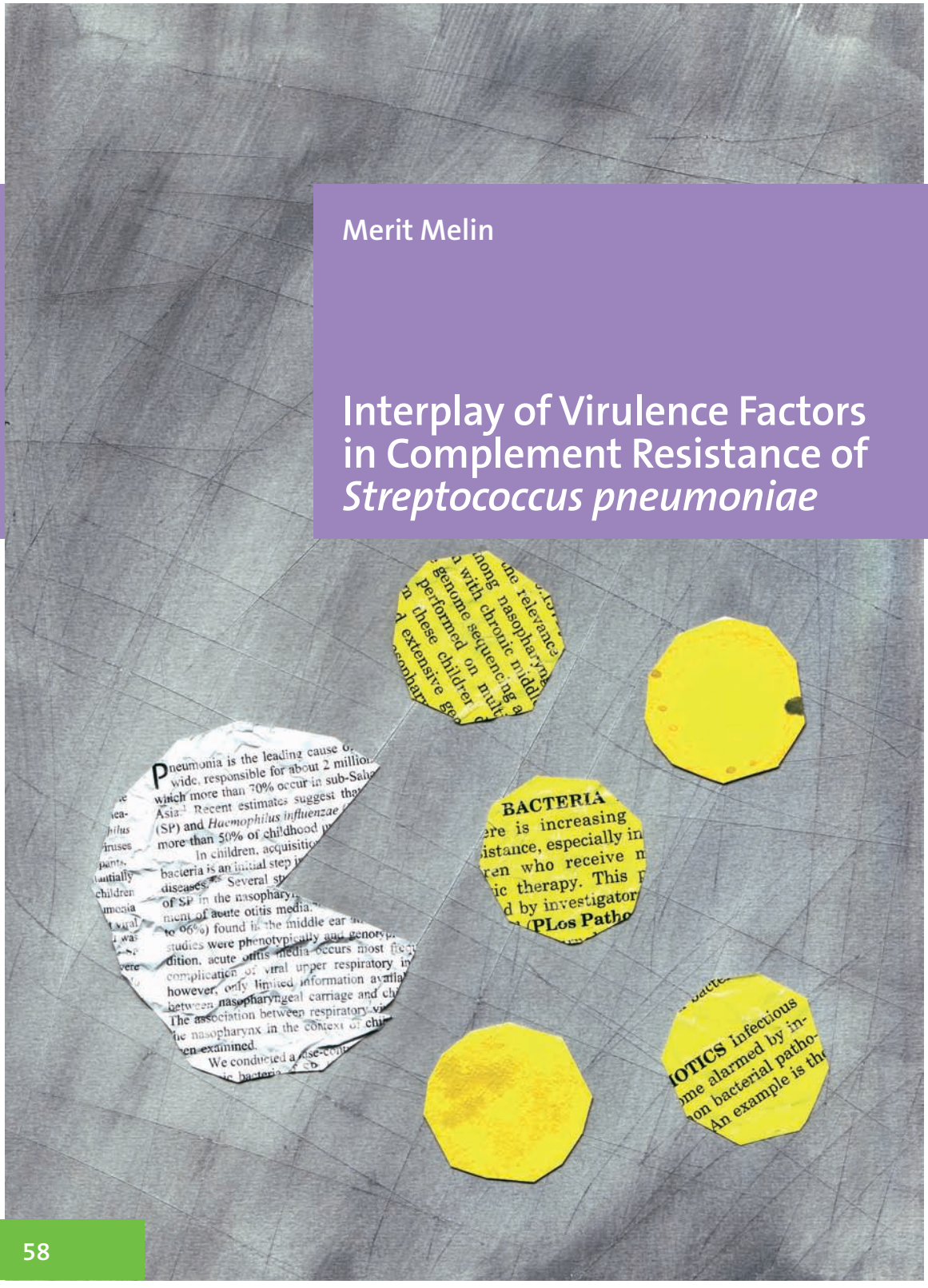
brought to you by CO
provided by Helsingin yliopiston digitaalinen a

RESEARCH

Merit Melin

Interplay of Virulence Factors in Complement Resistance of *Streptococcus pneumoniae*



RESEARCH 58

Merit Melin

Interplay of Virulence Factors in Complement Resistance of *Streptococcus pneumoniae*

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biosciences of the University of Helsinki in the Arppeanum auditorium, Helsinki University Museum, Snellmaninkatu 3, on May 6th, 2011, at 12 o'clock noon.

National Institute for Health and Welfare, Department of Vaccination and Immune Protection, Helsinki, Finland

University of Helsinki, Faculty of Medicine, Haartman Institute, Department of Bacteriology and Immunology, Helsinki, Finland

University of Helsinki, Faculty of Biological and Environmental Sciences, Department of Biosciences, Helsinki, Finland

Helsinki 2011

NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

© Merit Melin and National Institute for Health and Welfare

Cover photo: Merit Melin

ISBN 978-952-245-459-1 (printed)
ISSN 1798-0054 (print)
ISBN 978-952-245-460-7 (pdf)
ISSN 1798-0062 (pdf)

Unigrafia Oy
Helsinki, Finland 2011

Supervisors

Research Professor Helena Käyhty, PhD
National Institute for Health and Welfare
Department of Vaccination and Immune Protection
Helsinki, Finland

Professor Seppo Meri, MD, PhD
University of Helsinki, Haartman Institute
Department of Bacteriology and Immunology
Helsinki, Finland

Docent Merja Väkeväinen, PhD
National Institute for Health and Welfare
Department of Vaccination and Immune Protection
Helsinki, Finland

Members of the thesis advisory committee

Docent Kaarina Lähteenmäki, PhD
Research and Development
Finnish Red Cross Blood Service
Helsinki, Finland

Docent Mirja Puolakkainen, MD, PhD
University of Helsinki, Haartman Institute
Department of Virology
Helsinki, Finland

Reviewers

Docent Sakari Jokiranta, MD, PhD
University of Helsinki, Haartman Institute
Department of Bacteriology and Immunology
Helsinki, Finland

Docent Kaarina Lähteenmäki, PhD
Finnish Red Cross Blood Service
Advanced Therapies and Product Development
Helsinki, Finland

Opponent

Professor Birgitta Henriques Normark, MD, PhD
Karolinska Institutet
Department of Microbiology, Tumorbiology and Cell Biology
Stockholm, Sweden

Custodian

Professor Timo Korhonen, PhD
University of Helsinki, Faculty of Biological and Environmental Sciences
Department of Biosciences, Division of General Microbiology
Helsinki, Finland

ABSTRACT

Merit Melin. Interplay of Virulence Factors in Complement Resistance of *Streptococcus pneumoniae*. National Institute for Health and Welfare (THL), Research 58. 167 pages. Helsinki, Finland 2011.
ISBN 978-952-245-459-1 (printed), ISBN 978-952-245-460-7 (pdf)

Streptococcus pneumoniae (pneumococcus) is a normal inhabitant of the human nasopharynx. Symptoms occur in only a small proportion of those who become carriers, but the ubiquity of the organism in the human population results in a large burden of disease. *S. pneumoniae* is the leading bacterial cause of pneumonia, sepsis, and meningitis worldwide, causing the death of a million children each year. Middle-ear infection is the most common clinical manifestation of mucosal pneumococcal infections. In invasive disease, *S. pneumoniae* gains access to the bloodstream and spreads to normally sterile parts of the body. The progression from asymptomatic colonization to disease depends on factors characteristic of specific pneumococcal strains as well as the status of host defenses. The polysaccharide capsule surrounding the bacterium is considered to be the most important factor affecting the virulence of pneumococci. It protects pneumococci from phagocytosis and also may determine its affinity to the respiratory epithelium. *S. pneumoniae* as a species comprises more than 90 different capsular serotypes, but not all of them are equally prevalent in human diseases. “Invasive” serotypes are rarely isolated from healthy carriers, but relatively often cause invasive disease. Serotypes that are carried asymptomatically for a long time behave like opportunistic pathogens, causing disease in patients who have impaired immune defenses.

The complement system is a collection of blood and cell surface proteins that act as a major primary defense against invading microbes. Phagocytic cells with receptors for complement proteins can engulf and destroy pneumococcal cells opsonized with these proteins. *S. pneumoniae* has evolved a number of ways to subvert mechanisms of innate immunity, and this is likely to contribute to its pathogenicity. The capsular serotype, proteins essential for virulence, as well the genotype, may all influence the ability of pneumococcus to resist complement and its potential to cause disease. Immunization with conjugate vaccines produces opsonic antibodies, which enhance complement deposition and clearance of the bacteria. The pneumococcal vaccine included in the Finnish national immunization program in 2010 contains the most common serotypes causing invasive disease. Clinical data suggest that protection from middle-ear infection and possibly also from invasive disease depends largely on the capsular serotype, for reasons hitherto unknown.

The general aim of this thesis is to assess the relative roles of the pneumococcal capsule and virulence proteins in complement evasion and subsequent opsonophagocytic killing. The main question is whether differences between serotypes to resist complement explain the different abilities of serotypes to cause disease. The importance of particular virulence factors to the complement resistance of a strain may vary depending on its genotype. Prior studies have evaluated the effect of the capsule and virulence proteins on complement resistance of *S. pneumoniae* by comparing only a few strains. In this thesis, the role of pneumococcal virulence factors in the complement resistance of the bacterium was studied in several genotypically different strains.

The ability of pneumococci to inhibit deposition of the complement protein C3 on the bacterial surface was found to depend on the capsular serotype as well as on other features of the bacteria. The results suggest that pneumococcal histidine triad (Pht) proteins may play a role in complement inhibition, but their contribution depends on the bacterial genotype. The capsular serotype was found to influence complement resistance more than the bacterial genotype. A higher concentration of anticapsular antibodies was required for the opsonophagocytic killing of serotypes resistant to C3 deposition. The invasive serotypes were more resistant to C3 deposition than the opportunistic serotypes, suggesting that the former are better adapted to resist immune mechanisms controlling the development of invasive disease. The different susceptibilities of serotypes to complement deposition, opsonophagocytosis, and resultant antibody-mediated protection should be taken into account when guidelines for serological correlates for vaccine efficacy evaluations are made. The results of this thesis suggest that antibodies in higher quantity or quality are needed for efficient protection against the invasive serotypes.

Keywords: pneumococcus, complement, opsonophagocytosis, capsule

TIIVISTELMÄ

Merit Melin. Interplay of Virulence Factors in Complement Resistance of *Streptococcus pneumonia* [Virulenssitekijöiden vaikutus *Streptococcus pneumoniae* -bakteerin kykyyn suojautua komplementilta]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 58. 167 sivua. Helsinki 2011.
ISBN 978-952-245-459-1 (painettu), ISBN 978-952-245-460-7 (pdf)

Streptococcus pneumoniae (pneumokokki) on ihmisen nenänielussa esiintyvä bakteeri, jonka kantajista vain pienellä osalla ilmenee kliinisiä oireita. Pneumokokki on kuitenkin niin yleinen ihmisväestön keskuudessa, että sen aiheuttama tautitaakka on merkittävä. Vakaviin pneumokokkitauteihin (keuhkokuume, verenmyrkytys ja aivokalvontulehdus) menehtyy vuosittain yli miljoona lasta. Invasiivisessa taudissa pneumokokki leviää verenkierron kautta normaalisti steriileihin ruumiinosiin. Kantajuuden mahdollinen eteneminen taudiksi riippuu sekä pneumokokkikannan että isännän immuunipuolustuksen ominaisuuksista. Bakteeria ympäröivä polysakkaridikapseli on yksi tärkeimmistä pneumokokin taudinaiheuttamiskykyyn vaikuttavista tekijöistä. Kapseli suojaa bakteeria solusyönniltä ja voi vaikuttaa myös hengitysteiden pintasolukkoon kiinnittymiseen. *S. pneumoniae* lajina sisältää yli 90 erilaista kapseliserotyyppiä, joiden yleisyys taudinaiheuttajana vaihtelee. ”Invasiiviset” serotyypit ovat harvoin kannettuja, mutta aiheuttavat suhteellisen usein vakavia tauteja. Serotyypit, joita kannetaan oireettomasti pitkiä aikoja voivat aiheuttaa taudin henkilöillä, joiden immuunipuolustus on heikentynyt.

Komplementti koostuu joukosta veressä ja solujen pinnoilla esiintyviä proteiineja. Sillä on keskeinen merkitys elimistön puolustuksessa taudinaiheuttajia vastaan. Valkosolut, jotka ilmentävät pinnallaan erityisiä reseptoreja, tunnistavat ja nielaisevat komplementtiproteiineilla päällystetyt pneumokokkisolut estäen näin bakteerin leviämisen elimistössä. Pneumokokin taudinaiheuttamiskyvyn kannalta on ratkaisevaa, että se kykenee suojautumaan synnynnäiseltä immuunipuolustukselta. Kapselin serotyyppi, virulenssiproteiinit sekä kannan genotyyppi, eli ainutlaatuinen geenien yhdistelmä, vaikuttavat pneumokokin kykyyn vastustaa komplementtia ja aiheuttaa tautia. Rokotuksen seurauksena elimistö tuottaa komplementin kertymistä ja solusyöntiä tehostavia vasta-aineita. Vuonna 2010 kansalliseen rokotusohjelmaan sisällytetty pneumokokkirokote kattaa yleisimmät vakavia tauteja aiheuttavat serotyypit. Tutkimusten perusteella rokotteen suoja välikorvantulehdusta ja mahdollisesti myös invasiivista tautia vastaan vaihtelee olennaisesti serotyypistä riippuen, toistaiseksi tuntemattomista syistä.

Tämän väitöskirjatutkimuksen tavoitteena on arvioida pneumokokin kapselin ja virulenssiproteiinien merkitystä bakteerin kyvylle suojautua komplementilta ja solu-

syönniltä. Keskeisin kysymys on selvittää, ovatko serotyyppien välillä havaitut erot taudinaiheuttamiskyvyssä seurausta niiden erilaisesta komplementin vastustuskyvystä. On mahdollista, että virulenssitekijöiden merkitys pneumokokin kyvylle vastustaa komplementtia riippuu myös kyseisen bakteerikannan genotyypistä. Aiemmissa tutkimuksissa kapselin ja virulenssiproteiinien merkitystä komplementin vastustuskyvylle on arvioitu vertaamalla vain yhtä tai muutamaa pneumokokkikantaa. Tässä väitöskirjatyössä pneumokokin virulenssitekijöiden vaikutusta komplementin vastustuskyvylle selvitettiin tutkimalla virulenssitekijöiden merkitystä useissa genotyypiltään erilaisissa bakteerikannoissa.

Tutkimuksessa havaittiin pneumokokin kyvyn vastustaa komplementtiproteiinin C3 kertymistä bakteerin pinnalle riippuvan sekä kapseliserotyypistä että bakteerikannan genotyypistä. Pneumokokin pintaproteiinien (histidine triad, Pht) todettiin vaikuttavan komplementin vastustuskykyyn, mutta niiden merkitys riippuu todennäköisesti kannan genotyypistä. Tulokset osoittavat pneumokokin kapseliserotyypin olevan tärkein komplementin vastustuskykyyn vaikuttava tekijä. Kapseliserotyypillä havaittiin olevan merkittävämpi vaikutus kuin kannan genotyypillä. Komplementille vastustuskykyisten serotyyppien tappoon tarvittiin korkeampi pitoisuus kapselivasta-aineita. Nämä havainnot viittaavat siihen, että invasiiviset serotyypit ovat opportunistisia serotyyppejä sopeutuneempia vastustamaan immuunipuolustuksen keinoja rajoittaa invasiivista tautia. Serotyyppien väliset erot herkkyydessä komplementille ja solusyönnille tulisi huomioida rokotteiden tehoa arvioitaessa ja serologisia korrelaatteja koskevia ohjeita laadittaessa. Tämän väitöskirjatyön tulosten perusteella vastustuskykyisiltä serotyyppeiltä suojautumiseksi rokotteiden tulisi tuottaa korkeampi vasta-aineiden pitoisuus tai laadullisesti tehokkaampia vasta-aineita.

Avainsanat: pneumokokki, komplementti, fagosytoosi, kapseli

SAMMANDRAG

Merit Melin. Interplay of Virulence Factors in Complement Resistance of *Streptococcus pneumonia* [Virulensfaktorernas inverkan på förmågan hos *Streptococcus pneumonia* att skydda sig mot komplementet]. Institutet för hälsa och välfärd (THL), Forskning 58. 167 sidor. Helsingfors 2011.
ISBN 978-952-245-459-1 (tryckt), ISBN 978-952-245-460-7 (pdf)

Streptococcus pneumoniae (pneumokock) är en bakterie som trivs i människans näs-svalgrum. Pneumokocken orsakar kliniska symptom bara hos en liten del av dess bärare, men pneumokockens prevalens i människopopulationen är så hög att den ger upphov till en tung sjukdomsbörda. Globalt sett är *S. pneumoniae* den viktigaste bakteriella orsaken till blodförgiftning och lung- och hjärnhinneinflammation. Varje år avlider över 1 miljon barn till följd av pneumokocksjukdomar. Vid invasiv sjukdom sprids bakterien via blodcirkulationen till normalt sterila vävnader. Risken för att bärare av pneumokocker insjuknar beror på egenskaper i såväl bakteriestammen som i värdens immunförsvar. Polysackaridkapseln som omger bakterien är en av de viktigaste faktorerna som påverkar pneumokockens förmåga att orsaka sjukdom. Kapseln skyddar pneumokocken mot fagocytos och kan hjälpa den att fästa sig på slemhinnan. *S. pneumoniae* som art omfattar över 90 olika kapselserotyper, men alla är inte lika vanliga som sjukdomsalstrare. “Invasiva” serotyper förekommer sällan hos bärare, men de ger relativt ofta upphov till allvarliga sjukdomar. Serotyper som symptomfritt finns hos bäraren kan orsaka sjukdom hos patienter med nedsatt immunförsvar.

Komplementsystemet består av en grupp proteiner som uppträder i blodet och på cellytor. Det har en central roll i försvaret mot sjukdomsalstrarna. Leukocyter, som uttrycker vissa receptorer på sin yta, känner igen och sväljer pneumokockceller belagda med komplementproteiner och på det sättet förhindrar de att bakterien sprids i kroppen. Pneumokockernas förmåga att skydda sig mot det medfödda immunförsvaret är avgörande för deras förmåga att orsaka sjukdom. Kapselserotypen, virulensproteiner och stammens genotyp, dvs. den unika kombinationen av gener, påverkar pneumokockens förmåga att motstå komplementet och orsaka sjukdom. Vaccination stimulerar kroppen att producera antikroppar som främjar deposition av komplement och fagocytos. Pneumokockvaccinet som inkluderades i det nationella vaccinationsprogrammet år 2010 omfattar de vanligaste serotyperna som orsakar allvarliga sjukdomar. Kliniska undersökningar visar att vaccinets skydd mot mellanöreinflammation och möjligtvis också mot invasiv sjukdom beror på serotypen, av hittills okända orsaker.

Syftet med denna doktorsavhandling är att bedöma virulensproteinernas och kapselns betydelse för pneumokockens förmåga att skydda sig mot komplementet och fagocytos. Den centrala frågan är att utreda om skillnaderna mellan serotypernas förmåga att orsaka sjukdomar är en följd av att de har olika motståndskraft mot komplementet. Det är möjligt att olika virulensfaktorers relevans för pneumokockens förmåga att orsaka sjukdomar också beror på bakteriestammens genotyp. I tidigare forskning har kapselns och virulensproteinernas inverkan på pneumokockens resistens mot komplementet bedömts genom jämförelse av endast en eller några bakteriestammar. I denna undersökning görs en jämförelse mellan flera genotypiskt olika bakteriestammar för att utreda hur pneumokockens virulensfaktorer inverkar på bakteriens resistens mot komplementet.

Pneumokockens förmåga att förhindra att komplementproteinet C3 samlas på bakteriens yta beror enligt undersökningen både på kapselserotypen och på bakteriestammens genotyp. Resultatet visar att vissa av pneumokockens ytproteiner (histidine triad, Pht) påverkar motståndskraften mot komplementet, men deras påverkan beror på bakteriens genotyp. Enligt resultatet är kapselserotypen den viktigaste faktorn som påverkar resistensen mot komplementet; den har en större inverkan än bakteriestammens genotyp. I fråga om serotyper som är resistenta mot komplementet krävdes det en högre halt av antikroppar mot kapselpolysackarider för fagocytos. Dessa observationer tyder på att invasiva serotyper har anpassat sig bättre än opportunistiska serotyper för att motstå immunförsvarets mekanismer som kontrollerar invasiva infektioner. Skillnaderna mellan olika serotyper beträffande sensitiviteten till komplementet och fagocytosen borde beaktas vid bedömningen av vaccinernas effektivitet och utarbetandet av anvisningar om serologiska korrelat. Enligt resultatet av denna doktorsavhandling bör vaccin producera en högre halt av antikroppar eller kvalitativt effektivare antikroppar.

Nyckelord: pneumokock, komplement, fagocytos, kapsel

CONTENTS

Abstract ... 5
Tiivistelmä ... 7
Sammandrag ... 9
List of original publications ... 13
Abbreviations ... 13

1 INTRODUCTION ... 15

2 REVIEW OF THE LITERATURE ... 17
2.1 Pneumococcal carriage and disease ... 17
2.1.1 Exposure and colonization ... 17
2.1.2 Pneumococcal diseases ... 19
2.2 Host mechanisms of immune protection against *S. pneumoniae* ... 20
2.2.1 The complement system ... 20
2.2.2 The pathways of complement activation ... 21
2.2.3 Functions of the complement system ... 24
2.2.4 Regulation of complement activation ... 25
2.2.5 The role of complement in immune defense against pneumococci ... 25
2.2.6 The humoral immune response to pneumococcus ... 27
2.2.7 Cell-mediated immunity in pneumococcal infection ... 28
2.2.8 Opsonophagocytosis ... 29
2.3 Virulence factors involved in adhesion and early pathogenesis ... 30
2.3.1 IgA1 protease ... 31
2.3.2 Pneumococcal surface adhesin (PsaA) ... 32
2.3.3 Pneumococcal surface protein A (PspA) ... 32
2.3.4 Pneumococcal surface protein C (PspC) ... 33
2.3.5 Pneumolysin ... 35
2.3.6 Pneumococcal pili ... 35
2.3.7 The capsule ... 35
2.3.8 Biofilm formation ... 37
2.4 Virulence factors involved in complement inhibition in invasive disease ... 37
2.4.1 Complement evasion mechanisms of pathogenic bacteria ... 37
2.4.2 PspA ... 38
2.4.3 PspC ... 39
2.4.4 Pneumolysin ... 40
2.4.5 Pneumococcal histidine triad (Pht) proteins ... 40
2.4.6 The capsule ... 42

2.5 Prevention of pneumococcal disease 44
2.5.1 Polysaccharide vaccines 44
2.5.2 Conjugate vaccines 44
2.5.3 Serotype replacement 46
2.5.4 Protein vaccines 46
2.5.5 Resistance to antimicrobials 47

3 AIMS OF THE STUDY 49

4 MATERIALS AND METHODS 50
4.1 Bacterial strains 50
4.2 Serum samples 55
4.2.1 Sera used in the C3 deposition assay (I-IV) 55
4.2.2 Sera used in the factor H binding assay (I-II) 56
4.2.3 Sera used in the opsonophagocytic assay (II-IV) 56
4.3 Bacterial culture conditions 58
4.4 Complement C3 deposition assay (I-IV) 58
4.5 Factor H binding to bacterial cells (I-II) 59
4.6 Opsonophagocytic assay (II-IV) 59
4.7 Statistical methods 60

5 RESULTS AND DISCUSSION 61
5.1 Pneumococcal histidine triad (Pht) proteins may play a role in complement resistance of pneumococci (I) 62
5.1.1 Lack of Pht proteins resulted in increased C3 deposition on one of five pneumococcal strains (I) 62
5.1.2 Pht proteins do not inhibit complement by binding factor H (I) 65
5.2 The different abilities of capsular serotypes to resist complement deposition affect their susceptibility to phagocytic killing (II-IV) 67
5.2.1 The capsule affects phagocytic killing (II-IV) 68
5.2.2 The capsule affects complement resistance (II-IV) 70
5.2.3 Resistance to complement deposition is associated with decreased susceptibility to opsonophagocytic killing (II-IV) 76
5.3 Resistance to complement and opsonophagocytic killing is associated with serotype-specific mortality in invasive pneumococcal disease (III-IV) 79

6 CONCLUSIONS 82

7 ACKNOWLEDGMENTS 85

8 REFERENCES 87

LIST OF ORIGINAL PUBLICATIONS

I Merit Melin, Emmanuel Di Paolo, Leena Tikkanen, Hanna Jarva, Cecile Neyt, Helena Käyhty, Seppo Meri, Jan Poolman, and Merja Väkeväinen. Interaction of Pneumococcal histidine triad proteins with human complement. *Infect Immun* 2010 May;78(5):2089-98.

II Merit Melin, Hanna Jarva, Lotta Siira, Seppo Meri, Helena Käyhty, and Merja Väkeväinen. *Streptococcus pneumoniae* capsular serotype 19F is more resistant to C3 deposition and less sensitive to opsonophagocytosis than serotype 6B. *Infect Immun* 2009 Feb;77(2): 676-84.

III Merit Melin, Krzysztof Trzciński, Martin Antonio, Seppo Meri, Richard Adegbola, Tarja Kaijalainen, Helena Käyhty, and Merja Väkeväinen. Serotype-related variation in susceptibility to complement deposition and opsonophagocytosis among clinical isolates of *Streptococcus pneumoniae*. *Infect Immun* 2010 Dec;78(12):5252-61.

IV Merit Melin, Krzysztof Trzciński, Seppo Meri, Helena Käyhty, and Merja Väkeväinen. The capsular serotype of *Streptococcus pneumoniae* is more important than the genetic background for resistance to complement. *Infect Immun* 2010 Dec;78(12):5262-70.

The original articles are reproduced with the permission of the copyright holder, American Society for Microbiology, Washington, DC, USA. Previously unpublished data is also presented.

ABBREVIATIONS

AGS	agammaglobulinemic human serum
AOM	acute otitis media
ATCC	American Type Culture Collection
C3	third component of the complement system
C4BP	C4b-binding protein
CDC	Centers for Disease Control and Prevention
cfu	colony forming unit
CI	confidence interval
C-PS	C-polysaccharide
CR	complement receptor
CRP	C-reactive protein
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FcR	receptor for the Fc portion of immunoglobulins
FHL-1	factor H-like protein 1
GMC	geometric mean concentration
GMF	geometric mean fluorescence
HL-60	human promyelocytic cell line
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
IPD	invasive pneumococcal disease
kDa	kilodalton
KTL	Kansanterveyslaitos
LytA	major pneumococcal autolysin
mAb	monoclonal antibody
MAC	membrane attack complex
MASP	MBL-associated serine proteases
MBL	mannose-binding lectin
MLST	multi-locus sequence type
MOPA	multiplex opsonophagocytic assay
N/A	not applicable
NanA	pneumococcal neuraminidase A
ND	no data
NET	neutrophil extracellular trap
NHS	normal human serum
NMS	normal mouse serum

OD	optical density
OPA	opsonophagocytic assay
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
Pht	pneumococcal histidine triad protein
pIgR	polymeric immunoglobulin receptor
PRR	pattern recognition receptor
PS	polysaccharide
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
sIgA	secretory immunoglobulin A
SC	secretory component
SIGN-R1	ICAM-grabbing nonintegrin R1
Th	T helper cell
THYE	Todd-Hewitt broth supplemented with 5 % yeast extract
THL	National Institute for Health and Welfare
TLR	Toll-like receptor
USA	United States of America
WHO	World Health Organization
wt	wild-type

1 INTRODUCTION

Streptococcus pneumoniae is a gram-positive bacterium that colonizes the upper respiratory tract of healthy individuals. Occasionally, it breaks from its carriage habitat and causes infections ranging from acute otitis media to more severe diseases such as pneumonia, sepsis, and meningitis. *S. pneumoniae* is a heterogeneous species and it is divided into over 90 serotypes based on the structure of its capsular polysaccharide. The chemical structure of the polysaccharide capsule is a major determinant of pneumococcal virulence, as only a limited number of the serotypes accounts for the majority of invasive infections. Yet, clones sharing the same capsular serotype may have different abilities to cause disease, suggesting that other factors also contribute to their virulence.

The human complement system is an essential part of the innate immune system, our first line of defense against invading microbes. It can rapidly recognize and opsonize invading bacteria for phagocytosis. However, successful bacterial pathogens have in turn evolved ingenious strategies to overcome this part of the immune system. The ability of *S. pneumoniae* to evade complement attack is probably one of the key factors contributing to its pathogenicity. The thick gram-positive cell wall protects the bacterium from direct complement lysis, whereas a polysaccharide capsule shields it from phagocytosis by inhibiting complement deposition on the capsule and recognition of complement deposited on the bacterial cell wall. In addition to the capsular type itself, the combination of the capsule and other virulence factors of the pneumococcal strain accounts for pneumococcal virulence. Several pneumococcal virulence proteins have been found to act by inhibiting complement.

The purpose of this thesis is to assess the relative contribution of different pneumococcal virulence factors to the complement resistance of the bacterium. One of the questions is whether differences between pneumococcal serotypes to resist complement explain the different abilities of serotypes to cause disease. In addition, the potential function of a pneumococcal surface protein in complement inhibition in different genotypes was studied. By identifying factors and mechanisms whereby pneumococcus evades complement we will better understand how the microbe causes disease and how vaccines consisting of pneumococcal virulence factors could prevent it.

2 REVIEW OF THE LITERATURE

2.1 Pneumococcal carriage and disease

2.1.1 Exposure and colonization

Pneumococcus is a common component of the microbiota of the human upper respiratory tract. There are over 90 serologically distinct pneumococcal capsular serotypes. All of them are able to establish a carrier stage in humans. Variability in the colonization efficiency may exist since some serotypes are carried more frequently than others (38). The serotype affects nearly every aspect of pneumococcal pathogenesis as well as the nasopharyngeal carriage, which precedes disease and provides the reservoir for transmission of the organism (38).

Most children acquire pneumococcus in their nasopharynx during the first years of life, but the acquisition age and carriage rates vary by geographic location and population (113). The rate of acquisition is much slower in industrialized countries than in developing countries. At the age of two months, 9% of Finnish children are colonized by pneumococcus, and by the age of two years, the proportion of carriers is 43% (353). In The Gambia, 80% of infants carry pneumococcus by the age of two months (149). In Papua New Guinea all infants become carriers by the age of three months (246) whereas in Bangladesh the proportion of carriers at the age of four months is 50% (126). The prevalence of carriage increases during the first months of life. It starts to decrease after the age range of three to five years. The average carriage rates in different populations range from 40 to 50% in children and 20 to 30% in adults (114). In Finland, the frequency of carriage is only 3% in the adult family members of day-care children (208).

Young children may be simultaneously colonized with multiple capsular serotypes, which promotes horizontal gene transfer events and may lead to capsule switching between pneumococcal strains (57). However, epidemiological data suggest that different pneumococcal serotypes (or strains) compete with each other in colonizing human hosts. Acquisition of new serotypes in already colonized hosts was found to be weak in Danish day-care children (14). The duration of carriage is age-dependent (151). The first carriage in children lasts most commonly from 2.5 to 4.5 months, and decreases with successive pneumococcal serotypes and with age (96, 127). Prolonged carriage is seldom associated with progressive disease; a pneumococcal disease has been suggested to be associated with recent pneumococcal acquisition, where infection occurs within one month of acquisition of a new serotype (127, 206, 353).

The duration of carriage varies by capsular serotype (342) and is inversely correlated with the attack rate of invasive pneumococcal disease (Fig. 1). Capsular serotypes, which are carried for a short period and have a high attack rate, behave like “primary pathogens”: they affect previously healthy individuals and are associated with lower mortality. Meanwhile, serotypes that are carried for a long time behave like opportunistic pathogens. They infect patients with an underlying predisposition and are associated with more severe disease and higher mortality (341). This observation correlates with population-based studies, in which the risk of death from invasive pneumococcal disease (138) and pneumonia (377) was reported to be higher for serotypes with a high prevalence in carriage and low invasiveness.

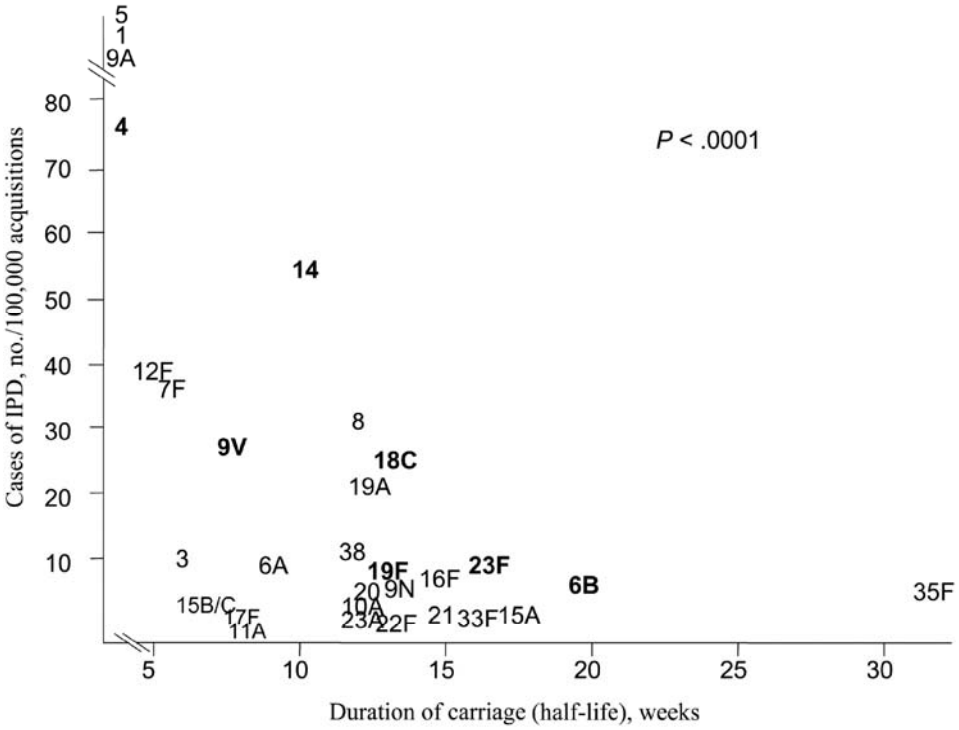


Figure 1. **Carriage duration and invasiveness of pneumococcal serotypes.** The median duration of carriage for each serotype was estimated from interval swabbing of the nasopharynx in a carriage study of two Oxford birth cohorts. The invasive disease incidence data were derived from national United Kingdom data from children under 2 years. Correlation between the attack rate (the incidence of invasive pneumococcal disease, IPD, per incidence of pneumococcal acquisition) and duration of carriage for each capsular serotype was statistically significant (P<.0001). From (342). Reproduced with the permission of the copyright holder, Oxford University Press.

2.1.2 Pneumococcal diseases

Nasopharyngeal colonization precedes pneumococcal disease, but symptomatic disease occurs in only a small percentage of persons who are colonized. Sometimes pneumococci invade adjacent sites and/or invade the bloodstream, causing disease. The clinical manifestations of pneumococcal infection can be classified into two major categories: *invasive* and *mucosal* infections. Mucosal infections of the middle-ear, the respiratory tract and the lungs are the result of the direct spread of the organism from the nasopharynx. Invasive disease follows usually haematogenous spread to normally sterile tissues. Invasive disease can also result from local spreading of the bacteria, for example from the lung to the pleural space or from the paranasal sinuses to the central nervous system. By far the most common form of pneumococcal disease worldwide is bacteremic pneumonia, the next most common form being pneumococcal meningitis, followed in order of decreasing incidence by blood stream infection (or sepsis) and otitis media (252, 280).

Acute otitis media (AOM) is a very common disease in industrialized countries among infants and young children, peaking during the age range of 6 to 18 months (188, 355). *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* are the predominant bacteria associated with otitis media, with pneumococcus being the most common causative agent (188). The bacteriology of acute **sinusitis** is similar to that of otitis media, with *S. pneumoniae* and/or *H. influenzae* being isolated in the great majority of cases (129). Prior respiratory tract infection of viral origin enhances the adherence of pneumococcus to tracheal cells and progression towards pulmonary infection (128, 283). Worldwide, an estimated 1.2 million people die of **pneumococcal pneumonia** each year (263). *S. pneumoniae* is considered to be the main cause of severe pneumonia among children and HIV-infected patients in the developing world (193). Pneumococcal pneumonia is also the most common cause of death due to infectious diseases in industrialized countries, contributing to morbidity and mortality, especially among elderly patients (106). **Invasive pneumococcal disease** (IPD) is diagnosed by positive blood or cerebrospinal fluid cultures. Spread of bacteria from the infection site to the bloodstream is relatively common in pneumococcal pneumonia. A positive blood culture can only be obtained from a minority of patients with pneumococcal pneumonia (15 to 30% of cases) (254), although a higher number of positive blood samples (44%) could be detected with PCR (358). **Pneumococcal meningitis** causes ~70,000 deaths annually, and other septic infections cause a similar number of deaths of young children in developing countries (263). Pneumococcal meningitis is associated with a higher mortality compared with other causative bacteria (22, 120), and neurological sequelae are common among those recovered (369, 391).

Pneumococcal infections affect specific **risk groups**. Conditions that increase the risk of serious pneumococcal disease are those that affect the immunological defense systems: reduced phagocytosis or ability to produce antibodies. Immaturity of the immune system in early life and waning immunity in the elderly predispose to pneumococcal diseases. Immunocompromising conditions include functional or anatomical asplenia, sickle cell disease, HIV, cigarette smoking, alcohol abuse, and the use of immunosuppressive drugs (61, 102, 196, 276, 294). Congenital deficiencies in the production of immunoglobulins increase the risk of developing pneumococcal infections (58). Individuals deficient in the early components of the classical or alternative pathway of complement have an increased susceptibility to recurrent pneumococcal infections (108, 168, 388).

2.2 Host mechanisms of immune protection against *S. pneumoniae*

The local host immunity has an important regulatory role in the trafficking of pathogens in the upper respiratory tract (113). A poor mucosal immune response might lead to persistent and recurrent colonization and consequent infection, whereas a brisk local immune response to the pathogen will eliminate colonization and prevent recolonization (114). In healthy individuals, the mucosal surfaces, with their epithelial cells and the secreted mucus, constitute a physical barrier that prevents pathogens from gaining access into deeper tissues. Once pneumococcus has crossed the first natural barriers of the host, it triggers activation of the host immune response (67). In the primary phase of infection, the innate immune defenses are of most importance, as induction of the antibody response takes time to develop. Antibody-mediated protection is important in preventing subsequent infections.

2.2.1 The complement system

The complement system is the major humoral part of innate immunity. Killing of microorganisms is one of its main functions (319). The complement system constitutes a critical link between the innate and acquired immunity by regulating B-cell- and T-cell-mediated immune responses (66). It consists of a series of proteins circulating in the blood and in tissue fluids. Many complement proteins occur in plasma as inactive enzyme precursors and some reside on cell surfaces. In response to the recognition of the molecular components of a microorganism, the complement proteins become sequentially activated, working in a cascade where the binding or activation of one protein promotes the binding or activation of the next protein in the cascade (199). The complement system can be activated through three different routes, triggered by various initiating proteins that recognize microbial ligands.

Classical pathway activation is initiated by C1q binding to Fc regions of antibodies on microbial surfaces, whereas the lectin pathway is activated directly by bacterial surface components in the absence of antibody-antigen complexes. The alternative pathway is initiated by the spontaneous hydrolysis of C3 and production of C3b, which can bind to pathogen (or host cell) surfaces. The pathways differ in the manner in which they are activated and ultimately produce key enzymes called C3 convertases (Fig. 2). The assembly of the C3 convertases is a pivotal step in the complement pathway. These enzymes cleave the C3 complement component to result in microbe-bound C3b which, in addition to its inactivated iC3b form, can be recognized by leukocytes.

2.2.2 The pathways of complement activation

The binding of antibody to its target antigen initiates activation of the complement system through **the classical pathway**. C1q of the C1 complex binds to appropriately spaced Fc regions of immunoglobulin molecules. It is important for IgG molecules to achieve a critical density on the surface in order to engage C1q and activate the C1 complex. Human IgG subclasses differ in their ability to activate the alternative pathway of complement. In general, the subclasses activate complement in the order IgG3 > IgG1 > IgG2. Because IgM is pentameric and each target-bound IgM can bind a C1q molecule, IgM is a more potent activator of the classical pathway than IgG. Natural IgM, which has specificity for microbial surface antigens and arises without prior exposure to the microbe, has a critical role in the immediate defense against bacterial infection (37). The classical pathway is also activated when members of the pentraxin family (which includes C-reactive protein, serum amyloid P component, and pentraxin 3) bind to surfaces and engage C1q (255, 313, 396). Classical pathway activation can also be initiated by the binding of certain pneumococcal polysaccharides to the specific adhesion molecule, (ICAM-3)-grabbing nonintegrin-related R1 (SIGN-R1), a lectin which is found in spleen macrophages (180). SIGN-R1 binds C1q directly, initiating the assembly of C3 convertase without the traditional requirement for antibody (180). Also, lipopolysaccharides of gram-negative bacteria can activate the classical pathway independently of antibodies (219). The C1 complex is formed by the association of the recognition unit C1q with a Ca^{2+} -dependent catalytic subunit consisting of C1s and C1r proteases (11). Formation of the C1 complex is followed by enzymatic cleavage of C4, resulting in exposure of an internal thioester bond in C4b (203), which can react readily with nucleophilic groups such as –OH to form an ester linkage or $–NH_2$ to form an amide linkage (92). In the next step in classical pathway activation, C2 binds to C4b deposited on the surface and is cleaved into C2a (and C2b). The remaining C4bC2a is the C3 convertase of the classical pathway.

The lectin pathway is triggered by the interaction of microbial carbohydrates with mannose-binding lectin (MBL) in the plasma and tissue fluids. MBL binds to polysaccharides rich in mannose and N-acetyl glucosamine residues, which are present on some microbial cells (111). MBL is assembled from identical polypeptide chains and bears structural similarity to C1q (93). As with the C1 complex of the classical pathway, the lectin pathway also consists of recognition molecules such as MBL and the ficolins and catalytic proteins, which are MBL-associated serine proteases (MASP-1, MASP-2, MASP-3) (368). MASPs are homologs of C1r and C1s of the classical pathway. MASP-2 is the main initiator of the lectin complement pathway by cleaving C4 and C2 to form the C4bC2a complex leading to further downstream complement activation (357).

The alternative pathway does not require initiation by antibodies and, thus, serves to protect the host from invading pathogens prior to the development of adaptive immunity. The alternative complement pathway is constitutively activated at low levels, but is only amplified when C3b binds to foreign surfaces. Spontaneous hydrolysis results in generation of an altered C3 molecule called C3(H_2O) capable of binding factor B (199). Once factor B associates with C3(H_2O), it undergoes a conformational change, which renders it susceptible to cleavage by the serine protease factor D, generating Ba and Bb (109, 210, 211). The Bb fragment remains attached to C3(H_2O) and, through its own serine protease domain, can cleave the C3a fragment from C3 to yield C3b. Cleavage of C3 results in a conformational change in the molecule and exposure of its internal thioester bond. Like C4b of the classical pathway, C3b can bind to –OH and –NH_2 groups on surfaces. Surface-bound C3b can then bind factor B, generate more C3 convertases, and thus set into motion an amplification loop.

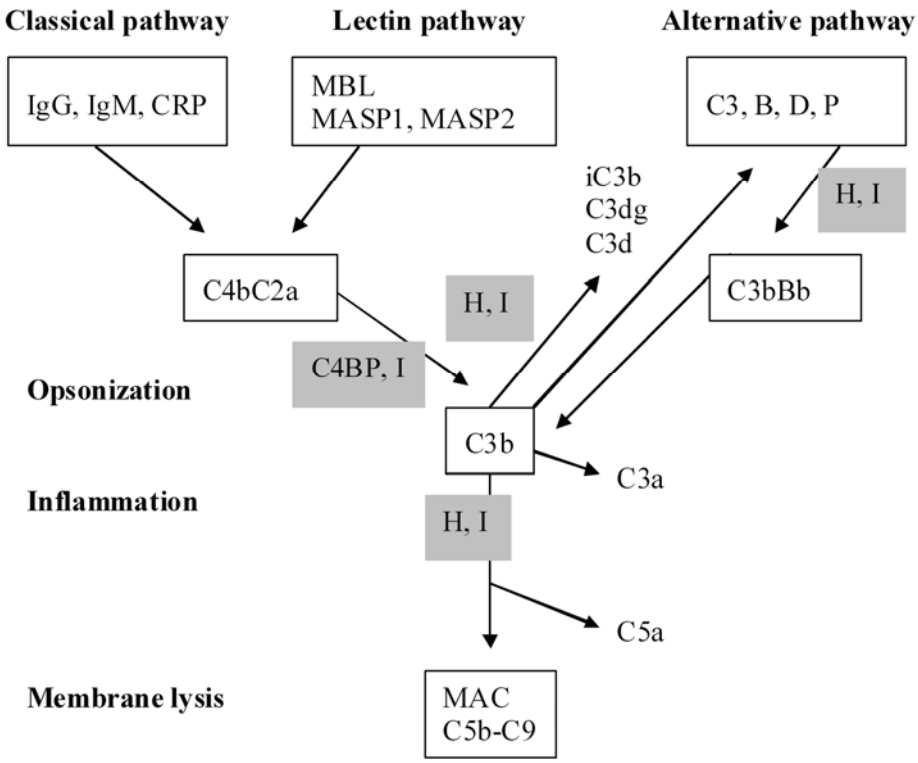


Figure 2. Complement activation pathways. Antibody-antigen complexes and CRP initiate activation of the classical pathway. Mannnose binding lectin (MBL) detects particular sugar units and activates the lectin pathway. The alternative pathway is activated by the binding of C3 to microbial surfaces. Activation initiated by any of the three pathways is further augmented by the activation of the alternative pathway: the amplification loop. Factor H (H) and C4b-binding protein (C4BP) act as cofactors for factor I (I)-mediated cleavage of C3b and C4b and dissociation of the C3 and C5 convertases.

Complement activation by each pathway generates a C3 convertase, which cleaves soluble C3 into two fragments: the anaphylatoxin C3a and the opsonic fragment C3b. C3b undergoes a conformational change that results in the exposure and disruption of a thioester bond linking Cys_{988} and Glu_{990} (354). The reactive carbonyl group of Glu_{990} attaches to the acceptor surface in a covalent ester or amide linkage with exposed carboxyl groups or with free amino groups of tyrosine residues, respectively (158, 204, 324, 334). Exposure of the reactive carbonyl group enables covalent binding of C3b to polysaccharides, amino sugars, or peptides exposed on the cell wall (157). With the assistance of various cofactors, e.g. factor H, surface-bound C3b is then sequentially processed, via the enzymatic activity of factor I, into smaller fragments (iC3b and C3d/C3dg). Importantly, C3b and its downstream

cleavage products remain covalently bound to the target surface and act as ligands for different complement receptors on phagocytic cells.

The terminal pathway of complement activation leads to formation of a membrane attack complex (MAC), which inserts itself into cell membranes and can cause osmotic lysis of target cells. MAC formation is initiated by the cleavage of C5 to C5a and C5b by C5 convertases of the classical pathway (C4bC2aC3b) or the alternative pathway (C3bBbC3b). The terminal components of complement, C6-C9, will sequentially build on C5b to form a C5b-9 complex into the cell membrane, where binding of more C9 molecules results in pore formation (362, 363).

2.2.3 Functions of the complement system

The complement system contributes to the host's defense against infection directly through its opsonic, inflammatory, and lytic activities and indirectly by enhancing antibody responses. Proteins produced by the complement pathways trigger inflammation (C3a, C5a, and C4a) and chemotactically attract phagocytes to the infection site (C5a). Formation of the terminal complex on the cell membrane causes lysis of gram-negative bacteria. Microbes sufficiently coated with C3 fragments are recognized by the different complement receptors (CRs) on various host phagocytes (Table 1). On the phagocytic cells, the CRs mediate attachment, engulfment, and killing of opsonized organisms (311). CR3 and CR4, expressed on neutrophils, monocytes, and macrophages, recognize iC3b, C3c, and C3dg molecules. They have high affinity for iC3b and lower affinity for C3b. CR1 is principally expressed on erythrocytes, monocytes, neutrophils, and B cells. It serves as the main system for the processing and clearance of complement-opsonized immune complexes. In contrast to CR3 and CR4, CR1 has high affinity for C3b and lower affinity for iC3b (32, 197), and it also binds C1q and C4b (192). CR1 mediates immune adherence and attachment to phagocytes, but is much less effective than CR3 and CR4 in promoting phagocytosis (122). Because CR1 has cofactor activity, it participates directly in complement activation by facilitating the factor I-mediated conversion of C3b to iC3b. This action of CR1 can down-regulate further complement activation (160). CR2 is a nonphagocytic receptor that binds C3dg and C3d and thereby influences activation and antibody production by B cells (88, 245). CRIg is found on macrophages residing in tissues, and it participates with CR3 in the removal of particles opsonized with C3b and iC3b from circulation (146).

Table 1. **Complement receptors (CRs)**

CR	CD proteins(s)	Main CR expressing cell types	Ligand(s)
CR1	CD35	Erythrocytes, Phagocytes	C3b, C4b
CR2	CD21	B lymphocytes	C3dg, C3d
CR3	CD11b/CD18	Phagocytes	iC3b
CR4	CD11c/CD18	Phagocytes	iC3b
CRIg		Tissue macrophages	C3b, iC3b

Adapted from Siber et al. 2008 (333).

2.2.4 Regulation of complement activation

To avoid overconsumption of and attack against host cells, while targeting microbial surfaces, the complement activation needs to be tightly regulated. This regulation occurs via both soluble (factor I, factor H, factor H-like protein 1 (FHL-1)), C4b-binding protein (C4BP)) and membrane-bound (CRIg, CD35, CD46, CD55, and CD59) complement regulatory proteins (Fig. 2).

Factor H is a plasma glycoprotein involved in the down-regulation of complement activation (240). Factor H controls complement activation on self cells by regulating the alternative pathway of complement activation in the fluid phase as well as on host cellular surfaces. Factor H binds to and inactivates C3b by serving as a cofactor for cleavage of C3b by factor I, dissociating the alternative pathway C3 convertase and by competing with factor B for binding to C3b (273, 375). The cleavage of C3b by factor I generates iC3b, C3dg, and C3d. If these molecules are attached to a microbial surface, they remain covalently attached to the organism and serve as ligands for complement receptors.

C4BP is a plasma protein that inhibits both the classical and lectin pathways of complement by acting as a cofactor for factor I-mediated degradation of C4b. It also accelerates the decay of the classical pathway C3 convertase (36). In addition, C4BP is a cofactor for factor I in the cleavage of C3b and may down-regulate the alternative pathway (35).

2.2.5 The role of complement in immune defense against pneumococci

The complement system is one of the most essential components of host defense against pneumococcus, which is shown by the severe infections suffered by individuals with complement deficiencies. Depletion of both alternative and classical pathways resulted in a lethal defect of intravascular clearance of bacteremic pneumococcal infection in a guinea pig model (154). In a murine colonization

model, C3-deficient mice lacking a functional complement system cleared pneumococcal colonization at the same rate as wild-type mice (370). In another study, nasopharyngeal colonization density was similar in complement-depleted and control mice, but in contrast to control mice, which remained healthy, the majority of complement-depleted mice developed sepsis (40). Until stable colonization is established, pneumococci reside in the luminal mucus (258), whereas the activities of phagocytic cells (macrophages as well as neutrophils) are likely to be confined to the tissues rather than taking place in the lumen (241). As most secretions are hypotonic, phagocytes probably do not survive with functional activity for long in the bulk fluid phase. However, the microenvironment close to the mucosal surface may permit phagocytic activity, as in the case of alveolar macrophages (241).

Alveolar type II epithelial cells and alveolar macrophages are known to synthesize and secrete complement proteins locally (72, 352, 373). Although local complement sources contribute relatively little to the total complement pool, which is mostly synthesized by the liver hepatocytes (72), a local source of complement along the alveolar epithelium may provide an important early clearance mechanism for pneumococci before immune cells are recruited and systemic complement can reach the lung. The concentrations of complement components present in mucosal secretions are usually well below those found in serum, and it is uncertain whether the classical or alternative complement pathway operates as a fully functional system in secretions (241, 373). Complement plays an important role in innate immune defense during the initial hours of pneumococcal infection within the lungs (184), where C3 probably acts as an opsonin for resident alveolar macrophages. Depletion of neutrophil-like cells did not increase the risk of sepsis in colonized mice, which implies a neutrophil-independent role for complement, specifically C3, in the prevention of a pneumococcal invasion following colonization by the rapid killing of invading bacteria (40). Development of otitis media and bacteremia in a chinchilla model was related to the ability of pneumococcal strains to resist C3 deposition, whereas the density of nasopharyngeal colonization was independent of the susceptibility of the strains to complement (322). However, all animal experiments with pneumococci need to be interpreted with caution because of the specificity of pneumococcus to humans.

Antibody to capsular polysaccharides or surface proteins of *S. pneumoniae* initiates the activation of the classical pathway by binding complement protein C1q on the Fc portion of IgG or IgM. The classical pathway can be activated not only by specific anti-pneumococcal antibodies, but with natural IgM (53, 404) and serum proteins detecting pathogen-associated structures and interacting with C1q (180, 400). The alternative pathway is activated even in the absence of adaptive immunity by the direct binding of C3b on the bacterial surface, but most importantly the alternative

pathway is responsible for augmenting C3b deposition (53). Amplification of complement activation by the alternative pathway is also essential when the activation has been initiated via the classical pathway (365). The classical pathway is considered the dominant pathway of complement activation in pneumococcal infection, because inhibition of the classical pathway, but not the alternative pathway, results in significantly reduced deposition of C3b on the bacterium (53). The lectin pathway was suggested to play only a minor role in complement activation in pneumococcal infection, because in a mouse model, the MBL pathway contributed little toward C3b deposition (53). In line with this finding, genetic MBL deficiency was found to be associated with only a small increase in susceptibility to pneumococcal disease in humans (314). However, in a study of African children with invasive pneumococcal disease, the authors found an association between infection by low invasive serotypes and certain common genotypes with MBL deficiency (367). Since pneumococcus has a thick gram-positive cell wall, it is resistant to direct complement lysis. The main function of complement in pneumococcal infection is opsonization of the bacterial surface with the C3 degradation products C3b and iC3b, which enables the intake of pneumococci by phagocytic cells with the help of complement receptors (105, 123, 312). Complement receptors CR1, CR2, CR3, and CR4 all play important roles in host defense against pneumococcal infection (296).

2.2.6 The humoral immune response to pneumococcus

Mucosal immunity has an important local regulatory role in the upper respiratory tract (113). In general, mucosal immunity matures earlier than systemic immunity, and is present from the age of 6 months (114). IgA, the major class of Ig in secretions, classically functions by interfering with microbial attachment to host tissues by binding to adhesion proteins on the bacterial surface (69). IgG and secretory IgA antibodies directed against capsular polysaccharides and surface-associated proteins have been observed in the saliva of children in response to colonization by *S. pneumoniae* (336, 337). Colonization also stimulates the production of systemic IgG responses to capsular polysaccharides and surface antigens such as pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumococcal histidine triad proteins (Phts), and pneumolysin (239, 293, 335, 346). Increased concentrations of serotype-specific antibodies against pneumococcal polysaccharides have been correlated with increased protection against carriage of a few common serotypes (121, 376), and antibodies against PspA in saliva have been associated with a decreased risk of acute otitis media (339). Pneumococcal carriage in a mouse model induced the production of mucosal IgA and systemic IgM in response to the capsular polysaccharide and IgG against PspA, which increased over time and correlated to reduced nasopharyngeal

pneumococcal numbers (300). Concentrations of serum antibodies to several pneumococcal surface proteins have been shown to increase during invasive infection (2, 215). Bacteremic infection incidence ordinarily peaks within the first 6 to 12 months of life and is thought to reflect the absence of type-specific anticapsular antibodies from the serum of young infants. However, susceptibility of adult volunteers to pneumococcal carriage correlated with serum IgG to PspA, but not with antibody to the homotypic capsular polysaccharide providing evidence for the role of antibody to this protein in preventing pneumococcal carriage by humans (237).

2.2.7 Cell-mediated immunity in pneumococcal infection

Although antibodies have been shown in multiple experiments to mediate protection against pneumococcal disease (217), the mechanisms of protection against colonization may be quite different from those involved in invasive pneumococcal disease. The decrease in the prevalence of pneumococcal carriage with increasing age beyond early childhood occurs in a largely serotype-independent manner (217). This suggests that the development of serotype-specific antibodies is not the only mechanism of immunity. The cellular compartment of immunity has been proposed to be the main effector in protection against colonization (229). The adaptive cellular immune response at mucosal surfaces to invading respiratory pathogens is based on the presence of antigen-specific CD4+ T lymphocytes. The significance of CD4+ T cells in clearance of pneumococcal colonization is supported by clinical findings. HIV infection is associated with a significantly increased risk of colonization and reduced time to new colonization (118). The increased risk of pneumococcal infection in HIV positive patients is inversely related to CD4+ T cell count (94, 117). The main populations of effector CD4+ T cells are type 1 T helper (Th1), type 2 Th (Th2), and type 17 Th (Th17) cells, typically producing interferon (IFN)-γ, interleukin (IL)-4, and IL-17, respectively. In a pneumococcal colonization model of mice, the clearance of bacteria from the nasopharynx required $CD4^+$ T cells and was independent of serotype-specific antibodies (232) or antibody to protein antigens, although the concentrations of antibodies to PspA and PsaA correlated with protection against colonization (359). Antigen-specific T-cell immunity, in the absence of antibodies, has been shown to be sufficient for protection against pneumococcal colonization in mice (361).

Pathogen-associated molecular patterns (PAMPs) are typically conserved structures found among microbes (277). These microbial components are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) expressed by cells of the innate immune system such as macrophages and dendritic cells (181, 198). TLR2 recognizes pneumococcal lipoteichoic acid and cell wall peptidoglycan (277), whereas TLR4 interacts with pneumolysin (230). Detection of pneumococci

by pattern recognition receptors initiates release of chemokines and cytokines, resulting in the recruitment and activation of leukocytes (277). TLR2 knock-out mice displayed increased disease severity in a pneumococcal meningitis model (95) and impaired clearance in a nasopharyngeal colonization model compared with wild-type mice (370). In murine pneumococcal pneumonia, TLR2 had only a modest contribution to the host response (195). Recognition of pneumolysin by TLR4 has been shown to have an inflammatory effect on macrophages *in vitro* (230). The role of TLR4 in pneumococcal infections appears to be localized to the airway surfaces, as the absence of TLR4 made no difference to survival rates and blood bacterial counts after intravenous infection of mice (27). However, in pneumococcal pneumonia, TLR4 mutant mice showed a reduced survival (42).

2.2.8 Opsonophagocytosis

Neutrophils, the most abundant group of leukocytes found in peripheral blood, are the most important effector cells mediating the opsonophagocytic clearance of pneumococci from circulation. Fc receptors, which detect antibodies bound to pneumococcal surface antigens, mediate neutrophil phagocytosis to some degree (323). However, opsonophagocytic killing of pneumococci is more strongly dependent on complement activation and phagocytosis mediated by complement receptors than direct antibody-mediated phagocytosis (323). Compared to the rate of phagocytosis in the presence of antibody alone, opsonophagocytosis of serotype 3 pneumococcus was accelerated sevenfold by the addition of complement proteins in fresh human serum (371). Opsonization with antibodies (IgG and IgM) that enhance deposition of opsonic C3 molecules is especially important in host protection against invasive pneumococcal infections. The standardized opsonophagocytic assay (OPA) measures the ability of antibodies to enhance the phagocytosis of encapsulated pneumococci (308). The *in vitro* opsonophagocytic activities of serum antibodies are believed to represent the functional activities of the antibodies *in vivo* and, thus, to correlate with protective immunity (175). Serum samples in which the levels of antibodies to capsular polysaccharides have increased after pneumococcal carriage often have opsonophagocytic activity against the homologous serotype (345).

It has been demonstrated in murine models that pneumococcal colonization of the upper respiratory tract triggers an acute inflammatory response characterized by a robust influx of neutrophils into the lumen of the paranasal spaces (258, 370). Data from a co-colonization mouse model (of *S. pneumoniae* and *H. influenzae*) suggest that successful clearance of pneumococci from the nasopharynx results from opsonization by complement, followed by phagocytosis by neutrophils, which are recruited to the mucosal surface (225). Alveolar macrophages are the first cells that combat pneumococci during early pneumonia (125) and the main cell population

that mediates mucosal responses in the lower airways. Alveolar macrophage phagocytosis of pneumococci and the clearance of bacteria from the lungs are enhanced by opsonization of bacteria with IgG and/or complement (124).
The acute inflammatory response may be ineffective in controlling the initial mucosal colonization (258), but it may however enhance the adaptive immune response and subsequent bacterial clearance (235). Prior exposure to live bacteria or intranasal immunization by killed pneumococcal whole cell antigen was found to confer protection in mice via interleukin 17A (IL-17A) produced by a subset of CD4+ T cells, so called Th17 cells (222). IL-17A-mediated protection against pneumococcal colonization results in the recruitment of neutrophils into the upper-airway lumen to clear bacteria, which has been found to occur both in the absence and in the presence of antibodies and complement (222, 407). TLR2-dependent activation of Th17 cells results in the recruitment of macrophages (during primary and secondary colonization) and neutrophils (during secondary colonization) into the upper airways of mice and subsequent clearance of pneumococci from the mucosal surface (407). Depletion of either IL-17A or CD4+ T cells was shown to block the recruitment of phagocytes required for the effective clearance of pneumococcal colonization (407).

2.3 Virulence factors involved in adhesion and early pathogenesis

Colonization of mucosal surfaces by pneumococcus is often a transient process, but it may also be an initial event in the progression to disease. A number of pneumococcal proteins are believed to promote colonization of the nasopharynx. Pneumococcus produces up to three different neuraminidase enzymes, NanA, NanB, and NanC, which cleave terminal sialic acids from host glycolipids and gangliosides, revealing new receptors for adherence or invasion (29, 64). NanA is the most strongly expressed neuraminidase and has a crucial role in biofilm production (275). Direct adhesive properties have been demonstrated in particular for PspC and pneumoccal pili-like appendages. Pneumococcal major autolysin (LytA) is a cell wall hydrolase, which degrades the peptidoglycan layer and is thus responsible for cell lysis under conditions in which biosynthesis stops, such as nutrient starvation or when the bacteria are treated with antibiotics (112). Many of the pneumococcal virulence factors have dual functions and contribute to both colonization and virulence in invasive disease (Table 2).

Table 2. Pneumococcal virulence factors

Virulence factor	Virulence functions	References
Neuraminidase	Reveales receptors for adherence by cleaving terminal sialic acids from the resiratory mucosa.	(29, 64)
Autolysin	Digests the bacterial cell wall and thereby releases inflammatory cell wall components.	(21, 112)
IgA1 protease	Cleaves human IgA1, which prevents antibodies from inhibiting adhesion.	(186, 285, 381)
Pneumococcal surface adhesin A	Aids binding to human epithelial cells.	(8, 31)
Pneumococcal surface protein A	Binds apolactoferrin, which may help in the acquisition of iron on mucosal surfaces. Inhibits complement C3 deposition.	(130, 133, 213, 296)
Pneumococcal surface protein C	Promotes adhesion to and translocation across epithelial layers. Inhibits complement C3 deposition.	(4, 132, 134, 169, 170, 292, 310, 405)
Pneumolysin	A multifactorial cytotoxin, which enhances inflammation and activates and consumes complement at a distance from the pathogen.	(234, 401)
Pili	Mediate adherence to respiratory epithelium.	(19, 23)
Capsule	Promotes colonization by helping the bacterium to escape from mucosal secretions to the epithelial surface. Protects from phagocytosis.	(162, 258)
Pneumococcal histidine triad proteins	Inhibits complement C3 deposition.	(269)

This list is not exhaustive and only selected examples are shown. For detailed descriptions of virulence factors, see the main text.

2.3.1 IgA1 protease

Pneumococcus possesses three large extracellular or surface-associated zinc-metalloproteinases, which are IgA1 protease, ZmpB, and ZmpC, which probably have different roles in the virulence of the bacterium (68). The mainly surface-associated IgA1 protease (Fig. 3) is known to specifically cleave the hinge region of human IgA1, the predominant class of immunoglobulin present on mucosal membranes (307). The cleavage of bound IgA1 produces bacterial surface antigens that are bound to Fab fragments, which prevents inflammation from being initiated

through host recognition of the Fc region of the antibody. IgA1 protease is important for the ability of pneumococcus to colonize mucosal membranes in the presence of secretory IgA antibodies (186, 285). Cleavage of serotype-specific IgA1 was shown to markedly enhance bacterial attachment to host cells (381). It appears that IgA1 protease has a dual role, which, on one hand, overcomes the inhibitory action of mucosal antibodies and, on the other, promotes adherence because bound Fab fragments may neutralize the inhibitory effect of negatively charged capsules upon adhesive interaction with host cells, allowing pneumococci to persist in the respiratory tract for extended periods (381). IgA1 protease is found in almost all clinical pneumococcal isolates, but the considerable antigenic heterogeneity of the protein renders it less attractive as a potential component of future vaccines (218).

2.3.2 Pneumococcal surface adhesin (PsaA)

Pneumococcal surface antigen A (PsaA) is a lipoprotein member of an ATP-binding cassette (ABC) transporter complex, which functions to transport manganese (90, 260). PsaA is a conserved antigen, which is present in all examined *S. pneumoniae* strains representing different capsular serotypes (248, 325). Mutations in PsaA have been shown to cause pleiotropic effects and reduce virulence in multiple infection models (31, 90, 233, 364). The role of PsaA as a potential pneumococcal adhesin was first demonstrated by the low adherence of the PsaA-deficient mutant to pneumocytes (31). Later, it was reported that PsaA-deficient mutants failed to adhere to human nasopharyngeal epithelial cells, and that antibodies to PsaA could inhibit the adhesion of wild-type pneumococci to the cells (309). PsaA is closely associated with the bacterial surface (Fig. 3), and it is assumed that the protein is exposed on the pneumococcal surface during carriage, when the capsule is relatively thin (205). E-cadherin was identified as the receptor for PsaA on human epithelial cells (8).

2.3.3 Pneumococcal surface protein A (PspA)

PspA is found in practically all clinical isolates of pneumococcus discovered to date (76). The majority of PspA molecules attach to the choline residues of lipoteichoic acids, which are anchored to the pneumococcal membrane (Fig. 3). A small fraction of PspAs might also be attached to the cholines of cell wall-associated teichoic acids (399). The PspA protein is remarkably variable at the sequence level. Based on sequence similarities, the proteins are divided to two major families, which can also be recognized serologically (152). PspA is required for full virulence in mouse models of pneumococcal disease (238), and it appears to be essential both for the ability of pneumococcus to colonize the nasopharynx and to cause lung infection and bacteremia (270). PspA binds the major iron transport protein lactoferrin, a

multifunctional protein that inhibits bacterial adherence and colonization on mucosal surfaces (130, 133). The binding of lactoferrin could aid the bacteria in the acquisition of iron on mucosal surfaces, or at the site of infection (130, 133). PspA has also been shown to bind apolactoferrin, the iron-depleted form of lactoferrin that has both bacteriostatic and bactericidal properties (330). The apolactoferrin that binds to PspA appears to be blocked from being able to kill pneumococci and even secreted forms of PspA could inhibit killing (330).

2.3.4 Pneumococcal surface protein C (PspC)

PspC is a multifunctional cell-surface protein known by several names, which reflect its different activities. The PspC family consists of 11 groups of polymorphic proteins with structural similarities, encoded by alleles of the same gene locus (164). Some of the alleles in the *pspC* locus encode choline binding proteins (Fig. 3) while some of the PspC proteins anchor directly to the cell wall (164). PspC has been shown to contribute to nasopharyngeal colonization and pneumonia (20, 271, 272), and it appears that PspC is important for translocation from the nasopharynx to the lungs and in crossing the blood-brain barrier (271). PspC has important roles in promoting adhesion to and translocation across epithelial layers (4, 132, 134, 292, 310, 405). Allelic forms have been named after their functional and binding characteristics.

Epithelial cells in the respiratory tract transport polymeric IgA from the basolateral surface to the lumen resulting in the presence of a polymeric immunoglobulin receptor (pIgR)/antibody complex on the apical surface (251). Cleavage of this complex permits the release of the antibody and a portion of the receptor that remains attached to the antibody (secretory component, SC). Unoccupied pIgR on the apical surface is recycled and returns to the basolateral surface for subsequent attachment to immunoglobulin. Apical recycling of the pIgR on the mucosal epithelium acts as an adherence mechanism for pneumococci. Some alleles of the PspC family can bind to pIgR, consequently enhancing transmigration of pneumococci from the apical to the basolateral face of the cells (51, 100, 405). PspC proteins choline binding protein A (CbpA) and secretory pneumococcal surface protein A (SpsA) bind the secretory immunoglobulin A (SIgA) via the secretory component (SC) (134, 344). Because excess of free SC and SIgA is present in the mucosal cavity, saturation of PspC by the binding of free SC or SIgA might decrease adherence mediated by the pIgR (99, 134). PspC has been shown to bind to host cells via secreted complement component C3 (310, 344). Clinical pneumococcal isolates, which are especially capable of binding to human lung epithelial cells, use PspC to bind to C3 on epithelial surfaces (305). Type II pneumocytes are known to synthesize and secrete complement component C3 (352),

providing a target for pneumococci to adhere to those cells via PspC. PspC also binds factor H (169, 170, 289). Factor H attached to the surface of pneumococci via PspC significantly enhanced pneumococcal adherence to host epithelial and endothelial cells (132). The integrin CR3 (CD11b/CD18) of human polymorphonuclear leukocytes was identified as the cellular receptor recognizing factor H (91). Pneumococcal entry into CR3-expressing epithelial cells via factor H is based on a two-step mechanism, where the initial contact of factor H-coated pneumococci is mediated by glycosaminoglycans expressed on the surface of human cells, and the pneumococcal uptake is mediated by integrins and depends on host signaling molecules (4).

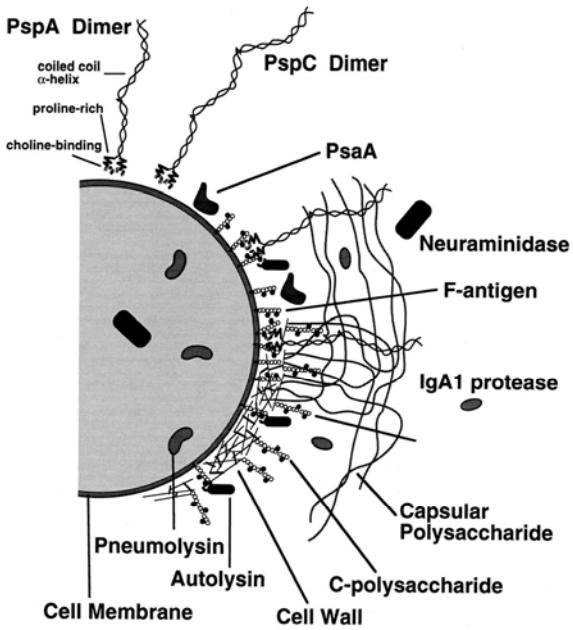


Figure 3. Pneumococcal surface structures. Diagrammatic representation of *S. pneumoniae* depicting several surface components for which roles in virulence have been described. PsaA, pneumococcal surface adhesin; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C. F-antigen is a choline containing lipoteichoic acid bound to the bacterial cell membrane (50), whereas C-polysaccharide is a teichoic acid linked to the cell wall. Originally published in (47) and reproduced with the permission of the copyright holder, Mary Ann Liebert Inc. Other important virulence factors not shown in the figure are pneumococcal pili and histidine triad proteins. Pili, expressed by many but not all pneumococci, are anchored to the cell wall. Electron microscopy images have indicated that pili 1 are shorter and cover most of the surface of the pneumococcus, while pilus 2 only exists as one or two copies, which extend further from the cell (19). Pneumococcal histidine triad proteins A, B, D and E have been detected on the bacterial surface by flow cytometry with antisera (2).

2.3.5 Pneumolysin

Pneumolysin is a major pneumococcal cytolysin that is produced by all known clinical isolates of *S. pneumoniae* regardless of serotype and genotype (173). It is a is a conserved thiol-activated, intracellular toxin (Fig. 3) that has multiple roles in the inflammatory reaction (150, 234). It contributes to the early pathogenesis of invasive pneumococcal pneumonia by facilitating intrapulmonary bacterial growth and invasion into the blood (317). The toxin is able to lyze eukaryotic cells; it disrupts the respiratory tract epithelium and inhibits cilial movement (41, 107, 316). Pneumolysin production is not a major determinant of successful nasopharyngeal colonization by pneumococci (318).

2.3.6 Pneumococcal pili

Some pneumococcal isolates express a pilus-like structure, which is able to extend beyond the polysaccharide capsule (23, 143). Pilus expression has been shown to mediate adherence to respiratory epithelium (19, 23). Pilus 1 is composed of three pilus subunits (RrgA, RrgB, and RrgC) (23, 143, 209). RrgA is considered to be the major pilus-associated adhesin, due to its localization at the tip of the pilus (257). The density of bacteria colonizing the upper respiratory tract of mice inoculated with piliated but RrgA-negative pneumococci was significantly less compared with the wild-type (257). The structural protein of pilus 2 is PitB, and adherence appears to rely solely on this pilus backbone protein (19). Pili 1 cover most of the surface of pneumococcus, while pili 2 are found in single copies and extend further from the cell (19). The pilus genes are not present in all the pneumococcal strains and clinical isolates: pilus 1 is present in less than 30% and pilus 2, only in less than 17% of the isolates (5, 19, 25, 249).

2.3.7 The capsule

The pneumococcal capsule is synthesized extensively during the exponential growth phase, and it is the thickest of the three surface layers of encapsulated pneumococci. With the exception of serotype 3, and possibly some other serotypes, the capsule is covalently attached to the outer surface of the cell-wall peptidoglycan (349). The capsular polysaccharide (PS) consists of repeating oligosaccharide units of two to eight monosaccharides. The majority of the PSs are simple linear heteropolymers, though some form more complex branched structures (7). The presence of the capsule can be verified by the Quellung reaction (15), in which bacterial cells are resuspended in antiserum that carries antibodies raised against the capsule. The test relies on the swelling of the capsule upon binding of homologous antibody, which can be visualized under a microscope.

Pneumococcus undergoes a reversible phase variation between opaque and transparent colony morphologies viewed in oblique light. Differences in colony opacity correlate with differences in virulence so that the transparent variants are more capable of colonizing the nasopharynx (190, 379, 382), whereas the opaque variants show increased virulence during systemic infections (190). Phase variation involves changes in the expression of surface molecules (190, 310). Most importantly, the opaque phenotype has more capsular polysaccharides compared with the transparent form (379). It has been demonstrated *in vitro* and *in vivo* that a phenotypic switch involving the down-regulation of the capsule exposes adhesion proteins in the nasopharynx for colonization, whereas up-regulation of capsule expression ensures abundant capsulation in systemic disease (135, 380). The capsule is one of the major virulence factors of *S. pneumoniae*. Loss of the capsule greatly attenuates virulence in animal models of disease (374). Pneumococcal strains that produce more capsule subunits *in vitro* are more virulent *in vivo* (226). All invasive clinical isolates are encapsulated, and the polysaccharide capsule is recognized to be the *sine qua non* virulence determinant for the bacterium.

The degree of encapsulation has not been previously shown to strongly impact nasopharyngeal colonization (228), but in a more recent study the serotypes most prevalent in carriage were found to be more heavily encapsulated (378). A minimum amount of the capsular polysaccharide is absolutely required for efficient nasopharyngeal colonization in mice (228). Pneumococcal variants expressing larger amounts of negatively charged capsule subunits per cell were less likely to adhere to surfaces coated with human mucus and more likely to evade initial clearance *in vivo* (258). Unencapsulated pneumococcal mutants retained their capacity for nasal colonization in mice, but at a reduced density and duration compared to their encapsulated parent strains (258). This impaired colonization could be attributed to the fact that unencapsulated mutants remain agglutinated within luminal mucus and are, thus, less likely to reach the epithelial surface, where stable colonization occurs. Expression of the capsule may also be beneficial upon subsequent invasion to the lungs and bloodstream, because it could affect susceptibility to trapping by neutrophil extracellular traps (NETs) (372) and killing by defensins (26). In humans, unencapsulated pneumococci are relatively frequently isolated from superficial infections such as AOM (136), but isolates that lack the capsule rarely cause invasive disease (141). Although a thick capsule could allow the bacterium to persist in the nasopharynx, lungs, and blood by protecting against host immune effectors, a large capsule could hinder the invasion process itself (301). Bacteria attached to the epithelium tend to be less heavily encapsulated than unattached pneumococci (135). It has been suggested that direct interaction with epithelial cells can promote invasion into underlying tissue and blood, and a thick capsule could disrupt this process (77, 78).

2.3.8 Biofilm formation

A biofilm is a highly structured, sessile microbial community characterized by bacterial cells attached to a surface or interface and embedded in a matrix of extracellular polymeric substances (74). Current evidence suggests that biofilm formation is a rather common feature among pneumococci that fits with some types of infections caused by this microorganism (AOM, meningitis), which have often been associated with the ability to form biofilm (65). In stable colonization pneumococci are likely to reside in the glycocalyx, which is the glue that holds the biofilm fast to the colonized surface (74, 258). The glycocalyx is a thin mucus-containing layer overlying epithelial cells, which consists of a complex of exopolysaccharides of bacterial origin and trapped exogenous substances found in the local environment, including nucleic acids, proteins, minerals, nutrients and cell wall material (74). Biofilm provides a physical barrier that enhances pathogen resistance to host defenses such as opsonization, lysis by complement, and phagocytosis (75). Pneumococci induced to form a biofilm *in vitro* had a greater propensity to cause pneumonia or meningitis, while planktonic cells from liquid culture were more effective in inducing sepsis (266). In the biofilm, pneumococcus exists in its unencapsulated form; the presence of a capsule reduces biofilm development (250). The reduced capsule is important in initial airway colonization both for facilitating the initial adherence and for promoting cell-cell interactions for biofilm formation (179). Many pneumococcal surface proteins, for example LytA, PspA, and PspC, seem to be important in biofilm formation, as mutants not expressing them had a decreased capacity to form a biofilm (250). Proteins involved in adhesion and virulence were more abundant under biofilm growth conditions (6). The ability of pneumococci to regulate capsule expression could play a fundamental role in the transition from the carrier state to invasive disease (366). It may be that, in the carrier state, pneumococcus constitutively adopts the sessile mode of growth in order to survive for long periods of time.

2.4 Virulence factors involved in complement inhibition in invasive disease

2.4.1 Complement evasion mechanisms of pathogenic bacteria

Immune-evasion strategies of pathogenic microorganisms are often focused on the complement system, which is the centerpiece of innate immunity and generally regarded as the first line of defense. Many, if not all, human pathogens have found ways to escape complement attack through a range of different mechanisms (201). In order to inhibit classical and lectin pathways, many microbes have developed the ability to bind to C4BP, which is a key inhibitor of these pathways, whereas protection from the alternative pathway is provided due to the capturing of factor H,

the major inhibitor of this pathway (34, 170). Another bacterial complement evasion strategy is to capture and inactivate the central molecule of all pathways of complement, C3. Furthermore, proteases with specificity for complement factors can efficiently destroy the bactericidal activity of serum.

The importance of complement in host defense against pneumococci is substantiated by the fact that the bacterium processes a variety of complement-evasion mechanisms. These activities involve interference with complement activation, inhibition of the deposition of opsonic C3 fragments, and perturbation of ligand-receptor interactions involved in opsonophagocytosis (333). The rigid gram-positive cell wall of *S. pneumoniae* prevents the bacteria from being lyzed by the membrane attack complex. The capsule prevents opsonophagocytosis by impairing CRP and antibody binding to the bacterial surface. It also reduces classical pathway activation, although the mechanism for this is not clear. The capsule is not the only strategy the pneumococcus uses to avoid complement and phagocytosis. Several pneumococcal proteins have been suggested to interfere with complement deposition, including PspA, PspC, and pneumolysin (172) and, more recently, pneumococcal histidine triad (Pht) proteins (269). In the following sections, the virulence factors that are considered to contribute to complement resistance of pneumococci are sidcussed in more detail.

2.4.2 PspA

PspA appears to protect *S. pneumoniae* from the host complement system and subsequent phagocytosis by macrophages (397, 399). Mutant strains of pneumococci not expressing PspA were cleared more rapidly from the blood of nonimmunized mice than the wild-type strain (49), whereas PspA^{-} pneumococci that were avirulent in normal mice were fully virulent in C3-deficient and factor B-deficient mice (365). Compared to wild-type pneumococcus, more C3b and iC3b were deposited onto the PspA^{-} strain, and the PspA^{-} strain also consumed more circulating host C3 than the wild-type strain (296-298, 365). Serum from a wild-type mouse supported more deposition of C3 onto the pneumococcal surface than serum from factor D- or factor B-deficient mice, and this decrease in the deposition of C3 *in vitro* correlated with impaired host resistance to infection (296, 297). PspA is suggested to inhibit the classical pathway (297) by interfering with the C1q initiation step, since the absence of PspA allows greater deposition of C1q and, thus, increased classical pathway-mediated C3 deposition (213). The anti-complement effect of PspA could be overcome by antibodies to PspA, allowing increased complement activation and C3 deposition on the bacteria (265, 297). Despite the wide diversity of PspAs, members from two PspA families had the same inhibitory effect on the activation and deposition of human C3 and on virulence in mice, when

the role of the PspA family was examined in isogenic strains with the same genotype (298).

2.4.3 PspC

As a complement evasion strategy, some pathogenic microorganisms recruit fluid-phase-soluble complement regulators to their surface. Factor H is a central host protein that is acquired by pathogens and attached to the pathogen surface to avoid complement-mediated killing. Most members of the PspC family can bind factor H, but the efficiency of factor H binding varies among pneumococcal strains (291). Factor H binding accelerates the degradation of C3b on the bacterial surface and inhibits the formation of new C3 convertases, which decreases the net C3b deposition (169, 170). Pneumococcus has also been shown to bind the complement inhibitor C4b-binding protein (C4BP) (89). C4BP is the major soluble inhibitor of the classical and lectin pathways. It inhibits the formation and accelerates the decay of the classical pathway C3 convertase (C4b2a) (83, 408). The binding of C4BP, like the binding of factor H, is dependent on PspC. However, C4BP binding seems to be restricted to certain serotypes, of which the strains of serotype 14 are the strongest binders and most of the high-binder strains harbor a specific allele of PspC (89, 404). The failure of *S. pneumoniae* to inhibit classical pathway activity by binding C4BP may be one possible explanation for the importance of the classical pathway in immunity to pneumococci.

The contribution of PspC to the virulence of pneumococci appears to depend on the strain. The deletion of PspC in the serotype 2, 3, and 19F strains did not significantly alter their virulence in a murine model of pneumococcal disease, whereas the virulence of a serotype 4 strain was reduced in both pneumonia and bacteremia models (183). Allelic variation in PspC structure may affect its interaction with the complement system. Comparison of several pneumococcal strains with different genetic backgrounds (genotypes) indicated that the effects of loss of PspC on C3 deposition are largely dependent on the genotype (403).

PspA and PspC may complement each other in their abilities to block the clearance of pneumococci by interfering with complement activation. Deleting PspA from a wild-type strain reduced virulence only moderately, but deletion of PspA from a strain lacking PspC caused a 10,000-fold decrease in virulence (47, 52). The deletion of PspC alone seemed to have little (290) or practically no effect (213) on complement deposition, while deletion of both PspA and PspC resulted in higher C3b deposition than when only one of the proteins was missing (213, 290). The results indicate that PspA and PspC markedly reduce complement deposition by inhibiting both the classical and alternative pathways. The absence of PspA and

PspC resulted in greatly increased immune adherence to human erythrocytes and improved the transfer of pneumococci from erythrocytes to phagocytes, which was suggested to be the mechanism for how PspA and PspC act in synergy to protect pneumococci from complement-dependent clearance during invasive infection (213).

2.4.4 Pneumolysin

The principal activities of pneumolysin have been viewed as the capacity to induce pores in cholesterol-rich membranes and the ability to activate complement, both of which play a role in virulence in an animal model of pneumonia (315). Pneumolysin contributes to complement resistance by quenching complement away from the pneumococcal surface (279) by activating the classical pathway of complement in the absence of antibodies (159, 279). Deletion of the gene encoding pneumolysin results in increased opsonophagocytosis of pneumococci via the classical pathway, which suggests that complement activation by the released toxin, pneumolysin, occurs at a distance from the organism and the original infection site, leaving less complement available to deposit on the bacterial surface (401). Comparison of single and double mutants showed that the two proteins work in concert; lack of both PspA and pneumolysin resulted in higher C3 deposition than either single mutation (401).

2.4.5 Pneumococcal histidine triad (Pht) proteins

Pht proteins were originally identified by immunoscreening from the genomic library of *S. pneumoniae* in search for surface-localized, immunogenic, and protective vaccine antigens (389). Pht proteins form a group of pneumococcal surface proteins characterized by a histidine triad motif, repeated five to six times in their amino-acid sequence (2). Four members of this family, with high sequence conservation between them, have been described: PhtA, PhtB, PhtD, and PhtE (131). PhtB and PhtD are most similar with 87% sequence homology, whereas PhtE shares only 32% similarity with the other Pht proteins (2). PhtA, PhtB, and PhtD have five histidine triads in their amino acid sequence; PhtE has six (2, 389). Results from antibody labeling and flow-cytometric experiments demonstrated that the Pht proteins are exposed on the surface of the encapsulated bacterium (131). The Pht proteins are well-conserved across the pneumococcal species (131, 406). The *phtD* gene was found to be present among all pneumococcal strains tested, and genes for PhtE, PhtB, and PhtA were found in 97%, 81%, and 62% of the strains, respectively (302). Fifty-four percent of the strains were found to carry all four *pht* genes in their genome (302). The *pht* genes are located in tandem pairs in the chromosome (2), but

their expression is controlled individually; *phtA*, *phtB*, and *phtE* each possess their own promoter, whereas *phtD* is under the control of an *lmb-phtD* operon (269, 274).

Deletion of one or two of the Pht proteins did not result in significant attenuation of the pneumococcal strain D39 in systemic disease compared with the wild-type pneumococcus, whereas the mutant lacking all four Pht proteins was completely avirulent (269). The observed functional redundancy is perhaps not surprising given the notable conservation of amino acid sequence between Pht proteins (2). In the pneumonia mouse model, the *phtA-phtB* deletion mutant, as well as the mutant lacking all Pht proteins, was significantly attenuated compared with the wild-type D39 (269). In contrast, single mutants lacking PhtA, PhtB, or PhtD in the TIGR4 strain were highly attenuated in the lung infection of mice (143). Several mechanisms of how Pht proteins may contribute to the virulence of pneumococcus have been suggested, and it is possible that they have pleiotropic functions. The high number of histidine residues in the histidine triads could imply that the Pht proteins play a role in DNA and/or metal binding (2) and could be involved in the adhesion and invasion process on mucosal surfaces where zinc concentration is low (274). The crystal structure of PhtA was reported to contain a zinc-binding motif (299), and the gene coding for PhtD is upregulated in zinc depletion as a member of an *lmb-phtD* operon (274). In another streptococcal species, *S. agalactiae*, the Lmb protein was shown to mediate bacterial attachment to human laminin, which is essential in bacterial colonization of damaged epithelium and translocation of bacteria into the bloodstream (350). A recent study suggests that Phts are regulators of metal homeostasis, Zn^{2+} and Mn^{2+} scavengers, with the function of storing and concentrating these divalent cations (302). As such, they may play a role in ion storage, particularly zinc, in order to make it available when the bacterium faces ion-restricted environments, as is the case during the early stages of infection (60, 140).

A role in immune evasion has also been postulated through suggestions that a fragment of PhtB (also termed PhpA) could cleave human complement component C3 (9, 155). It was suggested that PhtB would degrade soluble C3 proteolytically before deposition on the bacterial surface. This activity, however, has not been demonstrated directly. A more recent study suggests that Pht proteins would inhibit complement activation by binding factor H in a manner similar to PspC (269). Deletion of all four Pht proteins was reported to significantly increase C3 deposition on the bacterium, and the Pht proteins were reported to bind factor H (269). As in the mouse model of invasive pneumococcal disease, deletion of a single Pht protein had no effect on C3 deposition, suggesting overlapping roles for the Pht proteins in complement evasion (269).

2.4.6 The capsule

The pneumococcal capsule protects against phagocytic clearance by blocking the binding of antibodies directed against cell-surface antigens and deposition of opsonins (C3b and iC3b) on cell wall structures. The majority of C3b molecules are deposited on the cell wall, whereas capsule-bound C3b constitutes a minority of C3b molecules bound on encapsulated pneumococci (156). The capsule forms an inert shield that prevents the Fc region of IgG or opsonins bound to deeper cell-surface structures (for example, teichoic acids and cell-surface proteins) from interacting with their relevant receptors on phagocytic cells, thus impeding phagocytosis by Fcγ receptors and complement receptors. By reducing the binding of IgG and CRP on the bacteria, the capsule also inhibits activation of the classical pathway (162). The capsule may also inhibit bacterial interactions with nonopsonic phagocytic receptors such as mannose or scavenger receptors (12, 13). Although the neutrophil-mediated killing of pneumococci occurs in the absence of opsonins (378), neutrophil phagocytosis is markedly increased by opsonization of pneumococcus with complement (404). Complement deposition on isogenic *S. pneumoniae* TIGR4 mutants expressing different serotypes is correlated with neutrophil phagocytosis in the presence of complement, but differences in phagocytosis between strains were abolished when the bacteria were not opsonized with complement (163).

Reduced expression of the capsule results in greater access of antibodies and complement to the pneumococcal surface (228) and, hence, increased clearance by the immune system. However, the serotype of the capsule appears to be more important than its thickness to the ability of the capsule to inhibit complement deposition (163). Swap of the capsule type affected the accessibility of surface-bound complement and surface antigens of *S. pneumoniae* (1). Hostetter suggested that the antiphagocytic nature of the capsule might reside in its capacity to both permit C3b deposition and to enhance the proteolytic degradation of capsule-bound C3b to fragments that are recognized by phagocytes (iC3b) or fail to serve as ligands for phagocytic receptors (C3d) (155, 156). The absolute number of C3b bound on pneumococcal strains, in the study by Hostetter et al., did not correlate with the ranking order of how resistant serotypes are to opsonophagocytosis (155, 156). More recently, it has been reported that factor H bound to PspC is able to promote cleavage of C3b to iC3b (and C3d) by factor I, and that the effect of PspC on C3b/iC3b deposition depends on strain background and is largely independent of capsular serotype (402), implying that it is more likely that Hostetter's observations of C3b degradation were dependent on other factors than the capsular serotype.

Pneumococcal serotypes differ markedly in their capacity to cause disease (17). It has long been observed that certain serotypes, particularly 1 and 2, appear to be more "invasive" than other serotypes because they are not commonly carried by

healthy individuals (343, 351). An inverse correlation has been found between carriage prevalence and the invasiveness of pneumococcal serotypes (56). In order to compare the disease potential of different capsular types, the prevalence of serotypes among isolates from disease has been related to their prevalence in carriage (54, 136). Clones from certain serotypes were found to be significantly associated with invasive disease, while others appear to have a low disease-causing potential (54). Some serotypes, such as 1, 4, 5, 7F, and 14, are overrepresented among invasive isolates (54, 56, 137, 200, 320, 332, 342). Hanage et al. (2004) did not find significant differences among clones and serotypes in the risk of progression from carriage to AOM (136). In contrast, Shouval et el. (2006) reported a significant association of serotypes 1, 3, 5, 12F, 19A, and 19F with AOM (332). The differences in measures of serotype-specific potential to cause invasive or mucosal infections may be due to differences in disease potential between clones within the same serotype. The strong association of certain serotypes with invasive disease implies that the capsule type may be important in invasive disease, but in mucosal infections it may play a lesser role. A comparison of pneumococcal strains isolated from carriage and invasive disease suggests that carriage isolates are more heterogeneous, whereas clonal characteristics are likely to be advantageous for invasiveness (54, 304).

In addition to the capsular type, the combination of the capsule and the genotype of the pneumococcal strain was shown to be important and vary with the site of infection when capsule-switched mutant strains were studied in respiratory tract infections of mice (178). Although isogenic pneumococci expressing different capsule serotypes exhibit marked differences in murine virulence, non-capsular factors are also clearly important (182). In multi locus sequence typing (MLST) the allelic profile of a pneumococcal strain is obtained by sequencing internal fragments of seven house-keeping genes clones (101). Strains of different multi-locus sequence types expressing the same capsular serotype may have different virulence properties (326). Disease kinetics in mouse models varied significantly between clonal types within serotypes 1, 4, 6B, 7F, 14, and 19F (326). Differences in invasive disease potential between clones sharing the same serotype were also found among serotypes infecting humans. The propensity of clones to cause invasive disease in humans varied significantly among serotypes 3, 6A, 6B, 14, 19A, 19F, and 23F (320). Molecular epidemiological analysis has demonstrated that properties associated with a particular clonal type, in addition to capsular serotype, influence the potential of *S. pneumoniae* to cause invasive disease in humans. Certain clones have been particularly successful and have acquired multiple serotypes (55, 216). This supports the notion that other genetic factors influence the fitness of a pneumococcal strain. The relative contribution of the capsular type compared to other virulence factors in different pneumococcal diseases is still unclear. Since

considerable genetic diversity exists between strains, it is difficult to assess the significance of capsule versus other virulence factors. Recent studies using capsule-switched mutants show that protection against complement-mediated immunity varies between capsular serotypes even when expressed in an otherwise isogenic background (163).

2.5 Prevention of pneumococcal disease

2.5.1 Polysaccharide vaccines

Pneumococcal polysaccharide vaccines contain purified capsular polysaccharides from the serotypes most prevalent in industrialized countries. The first such vaccine included 14 serotypes and was licensed in 1977, and in the early 1980s the valence was increased to 23 serotypes. The vaccine serotypes represent 85 to 90% of the serotypes that cause invasive infections in the USA (Table 3).

The polysaccharides induce production of antibodies by stimulating clonal expansion of B cells by binding and cross-linking the B-cell receptor. Since polysaccharides are unable to recruit CD4+ T cell help through T-cell receptor recognition of peptide-major histocompatibility complex II, the antibody titers do not rise beyond the primary level after multiple exposures (139). The immaturity of B cells in young children makes them unresponsive to polysaccharide antigens, rendering such vaccines useless in children under 2 years of age (110). The PS-vaccine is effective in the prevention of bacteremia in adults. However, it does not offer protection against pneumonia, which is a more common manifestation of pneumococcal infection in elderly persons (153, 165, 331).

2.5.2 Conjugate vaccines

In the pneumococcal conjugate vaccines, capsule oligosaccharides are covalently conjugated to carrier proteins; e.g. a non-toxic mutant diphtheria toxin or protein D of *Haemophilus influenzae* (286). Conjugation of the polysaccharide to a protein carrier overcomes the problem of poor immunogenicity of the plain polysaccharide. The peptide part of the vaccine is recognized by T-cell receptors, which leads to a T-cell-dependent response: long-lived memory B cells, booster effect, and affinity maturation of the antibody response. Most importantly, the responses will take place in young children (110). The first 7-valent, pneumococcal conjugate vaccine was licensed in 2000 in the USA and in 2001 in Europe. The formulations of pneumococcal conjugate vaccines are listed in Table 3.

Table 3. Formulations of pneumococcal vaccines

Valence	Serotypes	Protein carrier	References
Polysaccharide			
23*	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 22F, 23F, 33F		(303)
Polysaccharide conjugated to protein carrier (PCV)			
7*	4, 6B, 9V, 14, 18C, 19F, 23F	CRM-197	(33, 103, 262, 387)
7	4, 6B, 9V, 14, 18C, 19F, 23F	OMPC	(187)
9	PCV7 + 1, 5	CRM-197	(79, 194, 236)
10*	PCV7 + 1, 5, 7F	PD, DT and TT	(383)
11	PCV7 + 1, 3, 5, 7F	PD	(286)
11	PCV7 + 1, 3, 5, 7F	DT and TT	(81, 223)
13*	PCV7 + 1, 3, 5, 6A, 7F, 19A	CRM-197	(59, 185)

The list is not exhaustive and includes licensed* vaccines and examples of vaccine formulations used in clinical trials. CRM-197, derivative of diphtheria toxin; DT, diphtheria toxoid; PD, *H. influenzae* outer membrane protein D; OMPC, meningococcal outer membrane protein complex; TT, tetanus toxoid.

The pneumococcal conjugate vaccine is efficacious in preventing serious forms of pneumococcal disease caused by the serotypes included in the vaccine (33, 79, 194, 262, 385). However, a case-control study in the USA suggests less efficient protection against invasive disease caused by serotype 19F (387). In The Gambia, the efficacy of the 9-valent vaccine was significant against invasive disease caused by serotypes 5, 14, and 23F, whereas despite good immunogenicity, the vaccine appeared to lack protective efficacy against serotype 1 (79, 321). The antibody concentration required for protection from colonization and mucosal infections is likely to be higher than for systemic disease (38). Marked variation was observed among pneumococcal serotypes in the efficacy of conjugate vaccines against AOM (103, 187, 286). The efficacy against pneumococcal AOM was good against 6B and 23F, but poor against 19F (103, 187), although the antibody concentrations after vaccination were the lowest for 6B and 23F and highest for 19F (97, 187). Lower efficacies against AOM caused by 19F have been reported in other studies as well (33, 261, 286).

2.5.3 Serotype replacement

In the first clinical trials of a tetravalent polysaccharide vaccine, it was found that if an individual was a carrier of a pneumococcal serotype represented in the vaccine, vaccination would not eliminate the carrier state, but if the individual was not a carrier, vaccination would reduce by half the likelihood of becoming one (227). The pneumococcal conjugate vaccines have since been shown to reduce acquisition of vaccine-type pneumococcal carriage, thus inducing indirect protection against carriage and disease in the unvaccinated, but also potential for replacement of nonvaccine types as causes of carriage and disease (103, 148, 161, 212). Although several studies have reported that pneumococcal conjugate vaccines decrease nasopharyngeal carriage of serotypes included in the vaccine (80, 82, 236, 264), the overall carriage rate has not diminished. This is because the ecological niche has been replaced by serotypes not immunologically related to the vaccine serotypes. In the USA, vaccine-type carriage has been replaced by non-vaccine-type carriage during the large-scale use of the vaccine since 2000 (77, 147, 161, 247, 281). Due to the ability of pneumococci to naturally take up DNA, they might readily escape selection pressure from vaccines directed at only a few serotypes by means of capsular switching. Well-known serotype switches include 23F to 19F, 23F to 14, 23F to 19B, 19A to 11, 14 to 19A, 14 to 9V, and 14 to 9A (87). Virulent, multi-drug-resistant pneumococcal clones circulating around the world seem to have the ability to change capsular type *in vivo* without loss of virulence (259).

2.5.4 Protein vaccines

While more than 20 pneumococcal serotypes may cause invasive disease, only a limited number of the capsule antigens can in practice be conjugated to a carrier protein. The use of pneumococcal protein antigens able to afford protection across the majority of serotypes is envisaged as a relevant alternative and/or addition to the use of polysaccharides in vaccines. Several proteins have been studied as potential future vaccine candidates. Virulence proteins are attractive targets, as they are essential to the bacteria and loss-of-function mutants are avirulent or show decreased virulence. Antibodies to surface-located proteins have the ability to both neutralize the virulence function and enhance phagocytosis by opsonizing bacterial surfaces. Pneumococcal surface proteins may provide cross-immunity regardless of serotype and have been investigated as carriers of polysaccharide antigens or used *per se* as potential vaccines. Proteins are immunogenic even in young children, and pure protein antigens would be less expensive since the costly conjugation process is unnecessary (44).

Antibodies directed against pneumococcal pilus structures were protective in mouse models of invasive disease against a pilus-expressing pneumococcal strain (115).

However, because pneumococcal pili are present in only a fraction of clinical isolates (19), they should be used in combination with other protective antigens to obtain broad-coverage, protein-based vaccines. Pneumococcal virulence proteins PspA, pneumolysin (278), PsaA (248), and Phts (2) are produced by virtually all clinically important pneumococcal strains, and proteins of the PspC family are also collectively expressed by most strains (164). Unlike *pspA* and *pspC* gene loci, the genes of PsaA (167) and pneumolysin (48) are relatively invariant. Phts are also highly conserved (2). The ability of a vaccine to prevent nasopharyngeal carriage is an important property, given that a reduction in carriage is likely to reduce transmission of pneumococci and acquisition of pneumococcal infection (278). Intranasal immunization of mice with PsaA was observed to reduce the bacterial load in nasopharyngeal carriage (43, 86). Also, protection against carriage increased to a greater degree in combination with PspA than after immunization with PsaA alone (43). Immunization with PspC has been effective in protection against nasopharyngeal carriage in mice (44). Immunization with PspA virtually eliminated nasal carriage (392). It has provided evidence of protection against otitis media in animal models (45, 384). PspA is also strongly protective against invasive disease (44), whereas immunization with PsaA failed to protect mice against systemic infection despite the protective effects against carriage (267). Immunization of mice with a PhtB antigen reduced nasopharyngeal colonization (406), and PhtB and PhtE antigens conferred protection against sepsis and pneumonia (131). In a nasopharyngeal colonization model and in a lung colonization model, Phts were found to be superior to PspA, PsaA, and PspC in terms of efficacy of protection and serotype coverage (119). Moreover, even natural anti-PhtD human antibodies transferred into mice in a passive transfer model demonstrated significant protection against lethal intranasal challenge (119). Previous studies in animal models suggest that immunization with a mixture of proteins provides the best protection from pneumococcal carriage or disease (43, 46, 267). The protective efficacy of the immunization of mice with pneumolysin, PspA, PspC, PhtB, and PhtE was assessed in an invasive-disease model (268), and a synergistic or additive effect was seen by using multiple proteins even where the individual proteins showed little value by themselves.

2.5.5 Resistance to antimicrobials

Pneumococcal infections were successfully treated with antibiotics until the emergence of the first penicillin-resistant strains of *S. pneumoniae* in the 1960s. Since then, the proportion of strains resistant to penicillin and a wide range of other antibacterial agents used in the therapy of pneumococcal infections has steadily increased (214). Multi-drug-resistant strains are becoming more common, some being susceptible only to vancomycin (386). The incidence of antibiotic resistance

varies geographically, most likely due to differing policies of antibiotic usage (166). Aggressive antibiotic treatment of AOM and viral infections of the upper respiratory tract is believed to be the major factor in the rise of antimicrobial resistance (116). The majority of pneumococcal strains causing AOM are not susceptible to penicillin in many countries (142). The highest rates of resistance to penicillin and erythromycin worldwide were found in serotypes included in the 7-valent pneumococcal conjugate vaccine. A significant decline in the rates of antibiotic resistance in the USA has been seen since the introduction of the vaccine (214). It has been feared that resistance to antimicrobial agents might spread to or develop in nonvaccine serotype strains. The high prevalence of penicillin-nonsusceptible strains in the non-vaccine serotypes, even before the introduction of pneumococcal vaccines, suggested that these clones could be potential candidates for replacement disease (284). Indeed, the incidence of infections caused by non-vaccine serotypes, especially by multi-resistant serotype 19A pneumococci, has increased (295). Inclusion of this serotype in the 13-valent conjugate vaccine is likely to significantly reduce the burden of disease due to serotype 19A.

3 AIMS OF THE STUDY

The capsular serotype, known virulence proteins, and the genotype, which is the unique combination of genes possessed by the pneumococcal strain, may all influence the ability of pneumococcus to resist complement and antibody-mediated protection and the resultant potential to cause disease. The general aim of the present study was to analyze the relative roles of the pneumococcal capsule and virulence proteins in evading complement attack and the subsequent opsonophagocytic killing. The importance of particular virulence factors to the complement resistance of a strain may vary depending on the genetic background. Prior studies have evaluated the effect of the capsule on complement resistance of pneumococci by comparing only very few strains (156, 163). Virulence proteins reported to influence complement inhibition have been assessed in only one or two strains (213, 269, 296-298, 365). In this thesis, I have aimed to assess the role of pneumococcal virulence factors in complement resistance of the bacterium by comparing their effects in several different genotypes.

The specific aims were:

1. to assess the effect of pneumococcal histidine triad (Pht) proteins on complement resistance by comparing wild-type and knock-out mutants of several genotypes

2. to study the ability of different pneumococcal serotypes to resist complement deposition and opsonophagocytosis by comparing clinical pneumococcal isolates that differ in their invasive disease potential

3. to compare isogenic mutants as well as clinical isolates sharing the same MLST, which express different capsular serotypes, to distinguish the relative roles of the serotype and genotype in resistance to complement and opsonophagocytosis

4 MATERIALS AND METHODS

The laboratory methods used in the studies of this thesis are listed in Table 4 and are described in more detail in the respective studies. An outline of the principal materials and methods is presented below.

Table 4. **Laboratory methods used in studies I-IV**

Method	Used in
Genetic methods	
Cloning of quadruple *pht* mutants	I
Cloning of capsule-switch mutants	IV
Multilocus sequence typing of pneumococcal isolates	II
Immunological methods	
Western blot analysis of expression of Pht proteins	I
Overlay assay for analysis of factor H binding to bacterial cell lysates	I
Enzyme immunoassay for PspA family typing	II
Enzyme immunoassay for binding of factor H to pneumococcal proteins	I
Opsonophagocytic killing assay	II-III
Flow-cytometric methods	
Surface expression of pneumococcal proteins	I
Complement C3 deposition on pneumococci	I-IV
Factor H binding to pneumococci	I, II

4.1 Bacterial strains

Clinical isolates of *S. pneumoniae* representing different capsular serotypes and genotypes as well as capsule-switched mutants and mutants lacking one or more surface proteins affecting the virulence of the bacterium were analyzed in this thesis.

Knock-out mutants lacking all Pht proteins were made in five different genetic backgrounds (Table 5). Previously prepared PspA^{-} and PspC^{-} mutants in the D39 background were analyzed along the Pht^{-} mutant D39 to assess the relative impact of the different proteins.

Table 5. Pneumococcal strains of study I

Strain	Serotype	Description	Reference/ source
D39	2	wild-type	(18)
D39 Pht^{-}	2	D39 derivative with *phtA*, *phtB*, *phtD*, and *phtE* deletions	study I
D39 PspA^{-}	2	JY182, D39 derivative with *pspA* deletion	(398)
D39 PspC^{-}	2	TRE108, D39 derivative with *pspC* deletion	(130)
D39 PspA^{-}PspC^{-}	2	TRE121, D39 derivative with *pspA* and *pspC* deletions	(130)
R36A	Rough	wild-type (ATCC[a] 27336), unencapsulated derivative of D39	(18, 202)
R36A Pht^{-}	Rough	R36A derivative with *phtA*, *phtB*, *phtD* and *phtE* deletions	study I
43	3	clinical isolate, naturally lacks *phtA*	clinical isolate, study I
43 Pht^{-}	3	43 derivative with *phtB*, *phtD* and *phtE* deletions	study I
4-CDC	4	wild-type (DS2382-94)	(308)
4-CDC Pht^{-}	4	4-CDC derivative with *phtA*, *phtB*, *phtD* and *phtE* deletions	study I
2737	19F	clinical isolate, naturally lacks *phtA*	clinical isolate, study I
2737 Pht^{-}	19F	2737 derivative with *phtB*, *phtD* and *phtE* deletions	study I

[a]ATCC, American type culture collection

Clinical isolates of selected serotypes were compared to explore potential differences between serotypes as well as variance within the serotypes in resistance to complement and opsonophagocytic killing (Table 6). The pneumococcal isolates of study II were characterized by multi-locus sequence typing (MLST), as described previously (101, 348). Isolates for study III representing different MLSTs were selected based on previously published data (136, 137). The mucosal isolates (nasopharyngeal carriage strains and middle-ear isolates from AOM) were collected for the FinOM Cohort Study from children under 2 years of age (188, 353). The invasive isolates came from blood cultures of Finnish children from the national infectious disease register (National Reference Laboratory for Pneumococcus,

National Public Health Institute, Oulu, Finland). Serotypes 1 and 5 are uncommon in Finland. The isolates of these two serotypes were selected from pneumococci isolated during clinical trials in The Gambia (3, 79). The clinical isolates were analyzed in parallel with the reference strains used in the opsonophagocytic assay. The reference strains of the single opsonophagocytic killing assay (OPA strains) (308) as well as of the multiplex OPA (MOPA strains) (39, 63) came from the Centers for Disease Control and Prevention, Atlanta. Capsule-switched mutants, which were constructed in three different genetic backgrounds, were compared with each other and with the donors of the capsule genes (Table 7). The invasive strains sharing the same MLST, but representing different serotypes, were isolated from Finnish children. They were obtained from the national infectious disease register.

Table 6. **Pneumococcal isolates of studies II and III**

Strain	Serotype	MLST	Sample type	Source	Study
MOPA1	1	ND[a]	invasive	SPEC-1 (L82006)	III
INV1-1	1	618	invasive	PVT0002	III
INV1-2	1	1331	invasive	PNI 0062	III
INV1-3	1	3579	invasive	PNI 0213	III
INV1-4	1	3574	invasive	PNI 0150	III
CAR1-1	1	910	mucosal	CH0089	III
CAR1-2	1	2084	mucosal	CH0053	III
CAR1-3	1	3570	mucosal	PVT 13506	III
MOPA4	4	ND	invasive	OREP-4 (DS2382)	III
INV4-1	4	176	invasive	IO11147	III
INV4-2	4	205	invasive	IO11512	III
INV4-3	4	205	invasive	IO12680	III
MOPA5	5	ND	invasive	STREP-5 (DBL5)	III
INV5-1	5	289	invasive	PVT0020	III
INV5-2	5	3313	invasive	PVT0027	III
INV5-3	5	3338	invasive	PVT0018	III
INV5-4	5	3339	invasive	PVT0026	III

Table 6 continues

Table 6 continues

Strain	Serotype	MLST	Sample type	Source	Study
MOPA6B	6B	ND	invasive	SPEC-6B (BG25-9)	III
6B OPA	6B	176	invasive	DS2212-94	II
6B NP	6B	506	mucosal	IOKOR 306-9	II
6B MEF1	6B	147	mucosal	IOKOR 1424-8	II
6B MEF2	6B	506	mucosal	IOKOR 248-7	II
6B MEF3	6B	497	mucosal	IOKOR 556-4	II
6B INV1	6B	138	invasive	io14922	II
6B INV2	6B	1518	invasive	io14924	II
6B INV3	6B	138	invasive	io14898	II
MOPA14	14	ND	invasive	STREP-14 (DS2214-94)	III
INV14-1	14	124	invasive	IO10163	III
INV14-2	14	700	invasive	IO626	III
INV14-3	14	9	invasive	IO12202	III
MEF14-1	14	134	mucosal	IOKOR1832-5	III
MEF14-2	14	156	mucosal	IOKOR2104-3	III
MEF14-3	14	307	mucosal	IOKOR392-1	III
MOPA18C	18C	ND	invasive	OREP-18C (GP116)	III
INV18C-1	18C	496	invasive	IO10148	III
INV18C-2	18C	1016	invasive	IO10162	III
INV18C-3	18C	1073	invasive	IO632	III
MOPA19F	19F	ND	invasive	SPEC-19F (DS2217-94)	III
19F OPA	19F	688	invasive	DS2217-94	II
19F NP	19F	309	mucosal	IOKOR 235-7	II
19F MEF1	19F	534	mucosal	IOKOR 1491-4	II
19F MEF2	19F	3760	mucosal	io16716	II
19F MEF3	19F	236	mucosal	io16650	II
19F INV1	19F	1081	invasive	io12089	II
19F INV2	19F	43	invasive	io12527	II
19F INV3	19F	43	invasive	io12690	II
MOPA23F	23F	ND	mucosal	EMC-23F (1212458)	III
INV23F-1	23F	37	invasive	IO11697	III
INV23F-2	23F	36	invasive	IO10783	III
INV23F-3	23F	440	invasive	IO10961	III
MEF23F-1	23F	37	mucosal	IOKOR46-1	III
MEF23F-2	23F	515	mucosal	IOKOR1101-8	III
MEF23F-3	23F	535	mucosal	IOKOR1617-3	III

[a]ND, no data

Table 7. Pneumococcal strains of study IV

Strain	Serotype	Capsule genes from/to	Source/ reference
Isogenic capsule-switched mutants			
TIGR4-R	rough	Janus cassette	(360)
TIGR4-1	1	GA07694	(378)
TIGR4-2	2	GA03901	Study IV
TIGR4-3	3	GA07650	Study IV
TIGR4	4	host strain	ATCC[a], (356)
TIGR4-4	4	TIGR4	(378)
TIGR4-5	5	501	(378)
TIGR4-6B	6B	NY00216	(360)
TIGR4-14	14	GA02190	(360)
TIGR4-19F	19F	GA71	(360)
TIGR4-23F	23F	TN82328	(378)
603-1	1	GA02290	(378)
603-5	5	501	(378)
603-6B	6B	host strain	(231)
603-14	14	GA02190	(378)
603-19F	19F	GA71	(378)
618-6B	6B	host strain	Study IV
618-14	14	GA02190	Study IV
618-19F	19F	GA71	Study IV
Donors of the capsule genes			
GA07694	1	TIGR4	ABC[b] (328)
GA02290	1	603	ABC[b] (328)
GA03901	2	TIGR4	ABC[b] (328)
GA07650	3	TIGR4	ABC[b] (328)
501	5	TIGR4, 603	(378)
NY00216	6B	TIGR4	ABC[b] (328)
GA02190	14	TIGR4, 603, 618	ABC[b] (328)
GA71	19F	TIGR4, 603, 618	(71)

Table 7 continues

Table 7 continues

Strain	Serotype	MLST	Source/ reference
Clinical isolates sharing the same MLST but different serotype			
199-19F	19F	199	(137)
199-6B	6B	199	(137)
156-14	14	156	(137)
156-9V	9V	156	(137)
156-19F	19F	156	(137)
162-14	14	162	(137)
162-9V	9V	162	(137)
162-19F	19F	162	(137)
66-23F	9V	66	(137)
66-9V	23F	66	(137)

[a]American Type Culture Collection, Atlanta; [b]Active Bacterial Core Surveillance of Centers for Disease Control and Prevention, Atlanta.

4.2 Serum samples

4.2.1 Sera used in the C3 deposition assay (I-IV)

Normal human sera (NHS) from adult subjects not immunized with a pneumococcal conjugate vaccine were used as a source of complement in the C3 deposition assay. Sera collected from 20 young adults were used in studies I and II, and sera from 10 voluntary adult donors (laboratory personnel) were used in studies III and IV (Table 8). The ethics committee of the National Public Health Institute, Helsinki, Finland, reviewed the protocol and approved the use of the sera. Sera were screened for antibodies to the corresponding capsular antigens and relevant protein antigens as previously described (338). NHS contained very low concentrations of antibodies to capsular polysaccharides, whereas concentrations of antibodies to pneumococcal surface proteins, especially to PhtD and PspC, were much higher (Table 8). A serum from a healthy adult immunized with PCV (immune human serum, IHS) was also used as the source of complement in study II. In studies I, II, and IV, each strain was analyzed twice with an agammaglobulinemic human serum (AGS), and in study I each strain was analyzed once with a pooled serum from naïve mice (NMS) (C57BL/6JRccHsd). Neither AGS nor NMS contained detectable concentrations of antibodies to the relevant protein and capsular antigens. All sera were divided into small volumes and stored at -70º C to preserve intact complement activity. Once thawed, the sample was used immediately as a source of complement for the C3 deposition assay.

Table 8. Concentrations of serum antibody to pneumococcal antigens in normal human sera (NHS) used in the C3 deposition assay[a]

Antigen	Study I	Study II	Study III	Study IV
Number of NHS analyzed				
	20	4	8	10
Antibody to capsular polysaccharides[a]				
1	N/A[b]	N/A	0.48 (0.15-1.46)	0.47 (0.18-1.19)
2	0.25 (0.14-0.44)	N/A	N/A	0.63 (0.21-1.89)
3	0.49 (0.27-0.87)	N/A	N/A	0.23 (0.67-1.93)
4	0.11 (0.05-0.23)	N/A	0.15 (0.06-0.40)	0.13 (0.06-0.30)
5	N/A	N/A	1.44 (0.44-4.69)	0.86 (0.26-2.82)
6B	N/A	0.16 (0.04-0.76)	0.28 (0.09-0.87)	0.33 (0.14-0.80)
9V	N/A	N/A	N/A	N/A
14	N/A	N/A	0.55 (0.12-2.51)	1.20 (0.23-6.17)
18C	N/A	N/A	2.79 (0.95-8.19)	N/A
19F	1.75 (0.85-3.60)	1.79 (0.59-5.40)	1.51 (0.80-2.84)	1.50 (0.93-2.42)
23F	N/A	N/A	0.77 (0.31-1.90)	0.79 (0.40-1.56)
Antibody to surface proteins				
PspC	11.7 (6.87-19.9)	4.68 (0.61-36.1)	12.8 (4.96-32.8)	9.98 (4.45-22.4)
PhtD	20.1 (14.0-28.8)	12.5 (8.3-18.9)	23.6 (11.3-49.2)	19.9 (10.6-37.6)
PspA-1	2.03 (1.14-3.64)	1.82 (0.23-14.1)	1.80 (0.77-4.20)	1.60 (0.82-3.12)
PspA-2	2.08 (1.40-3.08)	2.20 (0.21-22.9)	1.97 (1.08-3.57)	1.98 (1.26-3.10)

[a]Geometric mean IgG concentrations (µg/ml) with 95% confidence interval (CI). [b]N/A, not applicable.

4.2.2 Sera used in the factor H binding assay (I-II)

In the factor H binding assay, human serum from a healthy, unimmunized adult donor was heated at +56º C for 30 minutes to inactivate the heat-labile proteins of the complement system. The serum was divided into small aliquots and stored at -70º C before it was used as a source of factor H. Only one human serum was used in the assay; sera from different donors gave identical results in the factor H binding assay (data not shown).

4.2.3 Sera used in the opsonophagocytic assay (II-IV)

The functional activity of anti-capsular antibodies on opsonophagocytosis was assessed by analyzing the pneumococcal isolates with sera obtained from infants immunized with 4 doses of an 11-valent pneumococcal conjugate vaccine

manufactured by Sanofi Pasteur (394). Written consent was obtained from the parents of the children participating in the pneumococcal conjugate vaccine study before enrolment. The ethics committee of the National Public Health Institute, Helsinki, Finland, reviewed the protocol and approved the use of the sera in this study. In study II, each pneumococcal strain was analyzed with 4 to 5 different infant sera, and altogether 39 different sera were used. Each strain in study II was also analyzed with a control serum with high antibody concentration of serotype-specific antibodies, drawn from an adult vaccinated with the commercial PCV, Prevenar. Pooled sera, collected from the sera of 5 to 11 different infants each, were used in studies III and IV. The capsule type-specific antibody concentrations of the serum pools (Table 9) were calculated based on concentrations of the individual sera previously measured with enzyme immunoassay, EIA (393).

Table 9. **Concentrations of antibodies to capsular polysaccharides in the sera used in the OPA assay[a]**

Serotype	Low	Medium	High	Study
1	5.9 (3.2-10.8)	6.6 (5.3-8.1)	14.3 (10.5-19.6)	III, IV
2	NA[b]	NA	12.24[c]	IV
3	5.0 (2.8-8.7)	5.3 (2.6-10.9)	12.9 (9.7-17.3)	III, IV
4	1.4 (0.8-2.5)	6.2 (3.4-11.3)	14.8 (11.0-19.8)	III, IV
5	1.7 (1.0-2.8)	6.4 (4.6-9.0)	12.9 (8.5-19.6)	III, IV
6B	NA	5.8 (3.5-8.1)	59.6[d]	II
6B	1.2 (0.9-1.5)	5.8 (4.9-7.0)	14.9 (11.1-19.9)	III, IV
14	1.3 (0.7-2.4)	5.8 (3.4-9.8)	10.0 (5.2-19.1)	III, IV
18C	1.2 (0.8-1.8)	4.3 (2.5-7.5)	16.2 (10.0-26.1)	III, IV
19F	NA	6.6 (3.2-12)	56.8[d]	II
19F	5.2 (5.0-5.4)	7.2 (6.6-7.9)	15.5 (13.9-17.4)	III, IV
23F	1.3 (0.7-2.4)	5.8 (3.4-9.8)	10.0 (5.2-19.1)	III, IV

[a]Geometric mean IgG concentrations (µg/mL) of sera from infants immunized with an 11-valent pneumococcal conjugate vaccine, with 95% CI. [b]NA, not applicable. [c]Pneumococcal reference serum Lot 89-SF (287, 288). [d]Only one high serum was used for analysis.

4.3 Bacterial culture conditions

In flow-cytometric assays (C3 deposition and factor H binding), fresh bacterial cultures were used and the bacteria were cultured anew for each analysis because freezing might have different effects on the bacteria, depending on the strain background and serotype. Colonies with an opaque morphology on blood agar plates were inoculated into Todd-Hewitt broth supplemented with 0.5% yeast extract, THYE (in study II), or THYE supplemented with 5% (heat-inactivated) fetal bovine serum, FBS (studies I, III, and IV), and cultured to the early logarithmic growth phase to a defined turbidity. In study I, bacteria were cultured twice to the logarithmic phase using the first culture as an inoculum. Bacteria were harvested by centrifugation, and bacterial concentrations of the cultures were estimated based on their optical density from an OD_{620nm} vs. viable count curve. For the opsonophagocytic assay bacteria were prepared as described previously (308). In short, strains were cultured in THYE broth until the early logarithmic growth phase (in study II) or THYE supplemented with 5% FBS (studies II to IV). Glycerol, with a final concentration of 15%, was added to the cultures, which were divided into aliquots and stored at -70° C. The bacterial concentration of the frozen stocks was estimated by plating viable counts.

Culturing the bacteria in liquid medium has been shown to enhance expression of genes associated with invasive disease (266). The culture conditions, thus, favored opaque colony morphology. Culturing the bacteria in the presence of serum proteins in FBS was expected to further enhance expression of virulence factors essential to invasive disease. Transcriptional analysis of pneumococci isolated from the blood of infected mice has revealed adaptive changes in gene expression compared to different growth environments (272). For example, expression on laminin binding protein was increased in bacteremia compared to culture in defined semisynthetic casein liquid medium supplemented with 0.5% yeast extract (272). A genetic link between *phtD* and laminin binding protein has been reported (274), which suggest that the two are co-transcribed.

4.4 Complement C3 deposition assay (I-IV)

The major method used throughout all studies was the complement deposition assay, which measures the binding of C3 activation products on pneumococci. The bacterial concentrations were adjusted to 10^9 cells/mL in 20% serum diluted in buffer containing Mg^{2+} and Ca^{2+} ions in excess (these cations are necessary for the function of the classical and alternative complement pathways). A sample of

bacteria incubated with serum in buffer containing EDTA, which blocks both the alternative and classical pathways of complement activation by binding divalent cations, was used as a negative control in each analysis. Bacteria were incubated with the serum/buffer for 1 to 30 minutes to allow complement activation and opsonization, whereafter unbound serum proteins were washed away. Bound C3 molecules were detected by fluorescently conjugated antibody, which reacts with human C3c and with the C3c part in C3, C3b, and iC3b. The fluorescence intensity of the bacterial cells was measured by flow cytometry. Fluorescence intensity higher than 10 was considered positive for C3 binding. The percentage of positively staining bacteria (%>FL-10) was calculated for each sample in study II. Geometric means of fluorescence intensities were compared in studies I, III, and IV.

4.5 Factor H binding to bacterial cells (I-II)

The binding of factor H by pneumococcal cells was also measured by flow cytometry. Bacteria (10^9 cells/mL) were incubated with 30% heat-inactivated serum for 30 minutes, after which unbound serum proteins were washed away. The binding of factor H was detected by incubating the bacteria for 30 minutes with monoclonal murine anti-human factor H antibody 196X (study II) (177) or a commercially available antibody (Quidel, study I). The anti-factor H antibody was detected on the pneumococci with a secondary antibody that was conjugated to a fluorescent label. The geometric mean intensities of fluorescence were analyzed from each sample. The binding of factor H from serum was analyzed once in parallel with purified human complement factor H, used at a concentration of 90 µg/mL, which roughly corresponds to the factor H concentration in 30% human serum. Purified factor H and serum gave identical results, as has been reported previously (85).

4.6 Opsonophagocytic assay (II-IV)

The functional activity of serum antibodies against pneumococci was measured by a standard opsonophagocytic killing assay (308). The method uses HL-60 cells (promyelocytic leukaemia cells, CCL240, American Type Culture Collection, Rockville, USA), a human cell line that can be induced, e.g. by dimethyl sulfoxide (73), to differentiate into polymorphonuclear-like cells (neutrophil granulocytes) (308). The pneumococcal cells (500 colony forming units, cfu) were opsonized with serum antibodies in different dilutions, whereafter the polymorphonuclear cells (4 x 10^5 cells) were allowed to phagocytose the bacteria in the presence of 12.5% baby rabbit serum as source of complement. The assay was performed on microtiter

plates, and samples from each well were plated on THYE agar plates. The numbers of bacteria (cfu) that remained viable were counted and the results were interpreted as the serum antibody concentration that resulted in 50% of bacteria being killed as compared to the number of bacteria present in the control well in which only complement, but no antibodies, was present (50% reduction of cfu). If a proportion higher than 30% of the bacteria were killed by complement, in the absence of serum antibodies, the bacterial strain was cultured and analyzed again.

4.7 Statistical methods

Geometric mean fluorescence intensities (GMF) of C3 deposition on bacteria, with 95% confidence intervals (CI), were compared in studies I, III, and IV, whereas study II compared the averages of bacteria positive for C3 deposition with standard deviations. In studies I and II, the percentage of fluorescently labeled bacteria correlated well with the GMF of C3 deposition ($r=0.97$ and $r=0.98$, respectively, $p<0.001$, calculated from log-transformed GMF data). The data of study II were analyzed as the GMF of C3 deposition for this thesis. Because all bacterial strains bound factor H, GMF, not the percentage of bacteria positive for factor H binding, were compared in studies I and II. The geometric means of serum antibody concentrations (GMC) required for 50% opsonophagocytic killing with 95% CI were calculated in studies II to IV.

The student’s paired t-test was applied on log-transformed data in comparisons of wild-type and mutant pneumococci in study I. The student’s t-test was used in comparison of serotypes in study II, in comparison of mucosal versus invasive isolates and clinical versus reference strains in studies II and III, as well as in comparison of capsule donor strains with recipients in study IV. One-way ANOVA, followed by Tukey’s HSD (Honestly significant difference) post-hoc test, when appropriate, was applied for comparisons of serotypes in study III and for comparisons of isolates of the same genotype expressing different capsular serotypes or isolates from different genotypes expressing the same capsular serotype in study IV. Differences between individual strains within a serotype in studies II to IV were likewise compared by one-way ANOVA, followed by Tukey's HSD post-hoc test. Pearson’s correlation coefficient was calculated from antibody concentrations measured by EIA and from the geometric mean intensities of fluorescence for C3 deposition of individual NHS in studies I and IV. Pearson’s correlation coefficient was also calculated for complement deposition and opsonophagocytic killing using geometric means of C3 deposition and antibody concentration in studies III and IV. All the above-listed statistical analyses were performed on log-transformed data.

Pearson's correlation was also calculated between serotype-specific C3 deposition and the number of hydroxyl groups per polysaccharide, as well as between serotype-specific mortality of invasive pneumococcal disease (IPD) in patients aged 5 years or older (expressed as adjusted OR estimates) and serotype-specific C3 deposition, or antibody concentration required for 50% opsonophagocytic killing in study III. In all analyses, *p*-values less than 0.05 were considered to indicate a statistically significant difference.

5 RESULTS AND DISCUSSION

5.1 Pneumococcal histidine triad (Pht) proteins may play a role in complement resistance of pneumococci (I)

5.1.1 Lack of Pht proteins resulted in increased C3 deposition on one of five pneumococcal strains (I)

We assessed the influence of Phts on the complement resistance of pneumococci by comparing C3 deposition on the surface of wild-type and quadruple Pht-deficient mutant strain, which lacked all four Pht proteins (PhtA, PhtB, PhtD, and PhtE). Because the genetic background of the strain might affect the outcome, the influence of Phts was compared in five different strains. Sera from human donors were used as the source of complement in the C3 deposition assay. Antibody to pneumococcal antigens, present in NHS, may enhance C3 deposition (265). As antibody to Phts could potentially increase C3 deposition on the wild-type, but not the Pht^{-} strains, we also analyzed C3 deposition using AGS, which did not contain IgG to pneumococcal antigens.

The results suggest that Pht proteins could play a role in the complement resistance of pneumococci, but the relative importance of these proteins to the virulence of the pathogen depends on the genotype of the strain. Only one of the five Pht^{-} mutants bound significantly more C3 than its wild-type counterpart (Fig. 4). The result was the same with NHS and the AGS: deposition of C3 was two-fold on the Pht^{-} mutant 4-CDC compared to the wild-type, but no significant differences were found in C3 deposition between the wild-type and Pht^{-} mutant in any of the other genetic backgrounds (Fig. 4).

The relative influence of Pht-deletion, in comparison with deletion of PspA and/or PspC, on C3 deposition was assessed by comparing the D39 wild-type strain in parallel with the different mutants. We found that the PspA- and PspC-deficient D39 mutants bound more C3 than the wild-type strain when NHS was used as the source of complement, but the differences were not statistically significant. The results were similar with the AGS and NHS. Data from previous studies suggest that PspA, PspC, and pneumolysin work in concert. The lack of more than one of these proteins significantly increases the susceptibility of the strain to complement deposition (213, 290, 401) and virulence in animal models (268, 290, 401). The findings of this study were in concordance with the previous studies, as deposition of C3 was significantly increased on the PspA^{-}PspC^{-} double mutant compared to the wild-type D39, but no statistically significant differences in C3 deposition could be detected on the Pht^{-}, PspA^{-}, and PspC^{-} single mutants compared to the wild-type D39 strain (Fig. 4).

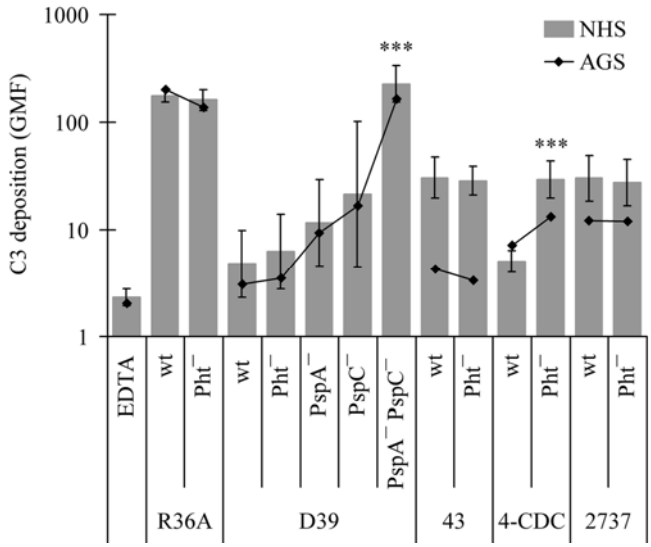


Figure 4. Deposition of complement protein C3 on the pneumococcal surface. Wild-type (wt), PhtABDE-quadruple mutant (Pht¯), and PspA¯ and/or PspC¯ mutant strains were compared by incubating the bacteria in 20% serum, after which deposition of C3 (C3b and iC3b) on the bacterial surface was detected with fluorescently labeled anti-C3 antibody and measured by flow cytometry. Each strain was analyzed with 6 (D39 strains) or 20 (all other strains) different normal human sera (NHS). A single analysis was performed with an agammaglobulinemic serum (AGS). Serum with 10 mM EDTA, which inhibits activation of complement via classical and alternative pathways, was used as a negative control. Geometric mean intensities of fluorescence (GMF) with 95% confidence intarvals are shown. ***$p<0.001$ (comparison of mutant with the corresponding wt strain by student's paired t-test, 2-tailed). The data originates from Fig. 4 and Fig. 5 of I.

In contrast to the results of this thesis, Ogunniyi et al. (2009) reported increased C3 deposition on the Pht¯ mutant D39 (269). One important difference in their methods compared to the present study was that they used mouse serum as the source of complement. Because no significant increase in complement deposition on the Pht¯ mutant D39 was seen with human sera, the strains of this study were also analyzed with a pooled serum from mice. The murine serum gave results similar to those observed with the human sera: increased C3 deposition was measured on the Pht¯ mutant 4-CDC and PspA¯PspC¯ mutant D39, but not on the Pht¯ mutant D39 (Fig. 5B of I). To confirm that deletion of Phts did not affect expression of other pneumococcal proteins, which could affect the complement resistance of the strains, expression of PspA and PspC on the surface of the pneumococcal strains was measured by flow cytometry. No significant differences in the surface expression of PspA or PspC were observed between the wild-type and Pht¯ strains. Ogunniyi et al.

(2009) confirmed, by using real-time RT-PCR, that deletion of any given *pht* gene had no effect on the level of transcription of any of the remaining *pht* genes (269), and that deletion of the suggested promoter region of the *pht* genes did not affect mRNA transcription of genes for PspC, PsaA, and pneumolysin, whereas transcription of *pspA* was three-fold lower in the *adcR* mutant (269). However, they did not mention if transcription or expression of PspA and PspC by the quadruple Pht mutant was characterized. It is possible that reduced expression of either PspA or PspC could have made their quadruple Pht^{-} mutant D39 strain more susceptible to complement.

It is possible that the genotype of the strains, rather than allelic variation of Pht proteins, partly explains why the lack of Phts affected complement resistance of one but not all of the studied strains. The lack of PspC impaired complement resistance of several but not all studied pneumococcal strains suggesting that allelic variation in PspC structure may affect its interaction with the complement system (402). The PspC family is heterogenic and different functions have been suggested for the alleles (164). For example, a particular allele has been associated with C4bp binding (89). The role of PspA in complement resistance of pneumococci has only been studied in two genetic backgrounds, D39 and WU2, where C3 deposition was increased on the PspA^{-} mutants compared with the respective wild-type strains (298, 365). Both PspA family 1 and family 2 proteins could inhibit C3 deposition on the WU2 strain (298). In contrast to PspC and PspA, the genes encoding Pht proteins are conserved (2, 131). Therefore, allelic differences in the Pht protein function are unlikely.

The efficacy of antibody-mediated protection has been shown to depend on the genotype of the challenge strains (2, 145, 306). PspA and PspC immunizations were efficient in protecting mice against challenge with the homologous pneumococcal strain D39, but vaccination with these antigens afforded little or no protection against a heterologous challenge with the 3-43 (serotype 3) and 4-CDC (serotype 4) strains (119). Previous studies have implied that the protection of immunized mice seems to be limited to the same PspA family (242, 306). It was suggested that allelic variation in the PspA and PspC molecules of the challenge strains could explain the different outcomes (119). Indeed, the PspA of D39 belongs to family 1 whereas strains 3-43 and 4-CDC have genes for family 2 PspA (119). The absence of protection by antibody to PspC against the 3-43 strain was understandable since this strain has a *hic* allele (PspC11) (169), which diverges from the PspC allele of D39. Previous studies reported that immunization with PhtB and PhtE protects mice against sepsis caused by WU2 and another serotype 3 strain (131), but not against D39 (268). Immunization with PhtD seemed to afford cross-protection against challenge by D39 as well as 3-43 and 4-CDC (119), which could be related to the

fact that PhtD is well conserved across pneumococcal strains (302). However, mice immunized with PhtA or PhtD were not protected against challenge with the homologous and highly virulent strain N4 (2). Similarly, immunization with PspA protected mice better from WU2 than from the homologous serotype 4 strain EF5668 (145). Passive immunization of mice with anti-PhtA antiserum afforded efficient protection against EF5668, which could indicate that Pht-proteins are more important than PspA to the virulence of EF5668 (2). We found that the serum antibody concentration to PspA correlated positively with C3 deposition on all the encapsulated wild-type strains except 4-CDC, whereas antibody to PhtD correlated positively, although weakly, with only 4-CDC. Since deletion of Pht proteins from D39 did not affect the susceptibility of D39 to complement, it is possible that the importance of Pht proteins in the D39 genotype is not as significant as it seems to be in genotype 4-CDC.

It would be interesting to compare how (or if) deletion of PspA or PspC would have affected on C3 deposition on the 4-CDC strain. Although lack of Phts increased C3 deposition on only one of the five strains, it is possible that deletion of PspA, PspC, or pneumolysin from the Pht^{-} mutants would have resulted in a synergistic effect on the susceptibility of the strains to C3 deposition. Also, deletion of Pht proteins from the EF5668 and other serotype 4 strains, as well as capsule switching of 4-CDC, would have been useful in the assessment of whether the serotype could be one factor affecting the role of Pht proteins to the strain. Because the genotype of the pneumococcal strain can have a significant influence on its virulence, it is important to study the effects of virulence factors using multiple, heterogeneous strains.

5.1.2 Pht proteins do not inhibit complement by binding factor H (I)

Factor H is a fluid-phase complement regulator, which pathogenic streptococci are capable of binding in a functionally active form, thus inhibiting C3 deposition on their surfaces (172). PspC has been shown to bind factor H and thus contribute to the ability of the bacterium to evade complement attack and opsonophagocytosis (84, 169, 170, 256). The PspC protein found in serotype 3 pneumococci, Hic, was first identified on the basis of its ability to inhibit complement activation by recruiting factor H on the bacterial surface (169, 170). The affinity of the PspC of *S. pneumoniae* D39 to factor H was later shown to be 23-fold stronger compared to Hic (220). In a previous study D39 was reported to bind mouse factor H, although to a lesser extent than human factor H (289). More recent studies have indicated that the interaction of PspC with factor H is species specific; clinical isolates bound only human factor H, not murine or any other factor H that were tested (rat, rabbit, horse or bovine) (221). Therefore it is not surprising that in mouse models of invasive disease the lack of PspC had little, if any, effect on strain virulence (30, 270, 271).

In line with the observation that factor H binding by PspC is species-specific, we found that the PspC^{-} mutant D39 was equally resistant to C3 deposition as the wild-type when murine serum was used as source of complement, whereas with human sera (NHS and AGS) more C3 was deposition on the PspC^{-} mutant compared to wild-type, although the difference was not significant.

To study the potential mechanism how Pht proteins could inhibit C3 deposition on pneumococci, different methods were used to measure potential binding of factor H, by pneumococcal strains or purified Pht antigens. Analysis of bacterial cell lysates with an overlay assay indicated that factor H binds specifically to the bands corresponding to the molecular size of PspC proteins (Fig. 6C of I). The size of PspCs ranges from 59 to 105 kDa (e.g. the PspC allele of D39 is a 75 kDa protein) (310), whereas PhtA, PhtB, PhtD and PhtE have molecular masses of approximately 92, 93, 94 and 115 kDa, respectively (2). Mutants lacking Phts exhibited factor H binding patterns identical to the wild-type strains, whereas PspC^{-} mutants bound no factor H (Fig. 6C of I). In the enzyme immunoassay the Pht antigens (PhtA, PhtB, PhtD and PhtE) did not bind factor H, whereas PspC was a strong binder of factor H (Fig. 6A of I). Flow cytometric analysis of factor H binding to pneumococcal cells further highlighted the importance of PspC for factor H binding, as no factor H binding was measured on the PspC^{-} mutant. All wild-type strains bound factor H and the lack of Pht proteins did not influence this interaction (Fig. 5). The Pht mutant 4-CDC strain bound factor H slightly but significantly less than the parent strain, whereas the D39 Pht mutant bound slightly but also significantly more than the wild-type. However, the difference between wild-type and Pht^{-} mutant 4-CDC was very small, and compared to the PspC^{-} mutant, it was still a strong binder of factor H. Ogunniyi et al (2009) reported that Pht proteins evade complement deposition in a similar mechanism as PspC and that Pht antigens would bind factor H (269). The finding by Ogunniyi et al. (2009) is controversial, as they used murine serum as source of complement, and clinical pneumococcal isolates have previously been reported to bind only human factor H (221). Furthermore, factor H binding is known to be almost completely dependent on PspC (402). The fact that direct binding of factor H to purified Pht antigens or Pht proteins in pneumococcal cell lysates was not seen in this study argues that factor H binding is not the main function of these proteins. Several different virulence functions in addition to complement inhibition have been reported for PspA and PspC. It is likely that Phts, too, have other attributes and their main virulence mechanism could be other than complement inhibition.

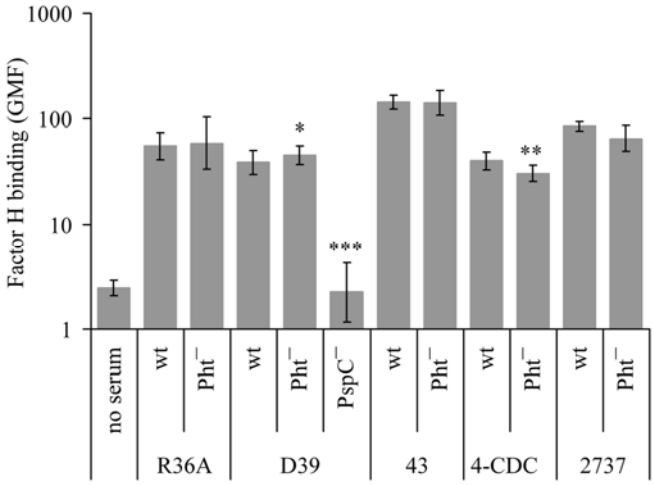


Figure 5. B**inding of factor H to w**ild-type (wt), PhtABDE- quadruple mutant (Pht⁻) and PspA⁻ and PspC⁻ mutant pneumococcal strains were analyzed by incubating the bacteria in heat-inactivated 30% human serum, after which binding of factor H to the bacterial cells was detected with fluorescently labeled antibody and measured by flow cytometry. Geometric mean intensities of fluorescence (GMF) from 3 repeated analyses with 95% CI are shown. *p<0.05, **p<0.01, ***p<0.001 (comparison with the corresponding wild-type by Student's paired t-test, 2-tailed). The data originates from Fig. 6B of I.

5.2 The different abilities of capsular serotypes to resist complement deposition affect their susceptibility to phagocytic killing (II-IV)

The outcomes of conjugate vaccine trials suggest that protection provided by immunization is suboptimal against serotypes 1 and 19F in invasive disease (79, 321, 387) and against AOM caused by 19F (33, 103, 187, 286). To find out reasons for the lower efficacy of conjugate vaccines to certain serotypes, we compared the ability of pneumococcal serotypes to resist opsonophagocytic killing and complement deposition and explored whether the amount of C3 deposited on the surface of the bacterium reflects its sensitivity to opsonophagocytosis. Several clinical isolates representing each capsular serotype were analyzed. The relative influence of the capsular serotype, compared to the strain background, on opsonophagocytic killing and C3 deposition was further evaluated by comparing isolates which share the same genotype but express different capsule types. Isogenic capsule-switched mutants and natural capsule switch variants of the same pneumococcal strain (which share the same MLST) were compared.

5.2.1 The capsule affects phagocytic killing (II-IV)

In the opsonophagocytic assay the concentration of antibodies required for killing pneumococci varied depending on the capsular serotype (Fig. 6a, b). A six-fold concentration of capsule antibodies was required for killing of serotype 19F clinical isolates compared to 6B isolates. Also, killing of clinical isolates of serotypes 1 and 5 required a significantly higher anti-capsular antibody concentration compared to serotype 23F. The antibody concentrations required for opsonophagocytosis in studies II (6B and 19F isolates) and III (other clinically relevant serotypes) cannot be directly compared because the studies used different serum sources and because the long term reproducibility of the assay is not optimal. To assess the sensitivity of serotypes 6B and 19F relative to other serotypes, reference strains of the opsonophagocytic assay were analyzed. Serotype 19F reference strain required a three-fold concentration of capsule antibodies compared to the serotype 1 and 5 reference strains for opsonophagocytic killing. This indicated that serotype 19F was the most resistant of the serotypes. There was little variation in the sensitivity to opsonophagocytosis between clinical isolates within serotypes, suggesting that the capsule type could be more important than the genotype for determining resistance to opsonophagocytosis. This finding was further supported by the observation that the concentration of anti-capsule antibodies required for killing isogenic strains or clinical isolates sharing the same genotype depended more on the capsule type they expressed and less on the genotype (Fig. 6c,d). Irrespective of the strain background, serotypes 1 and 19F required the highest concentrations of anti-capsular antibodies for opsonophagocytosis. The results clearly indicate that the concentration of type-specific antibodies required for opsonophagocytic killing is linked to the capsule type.

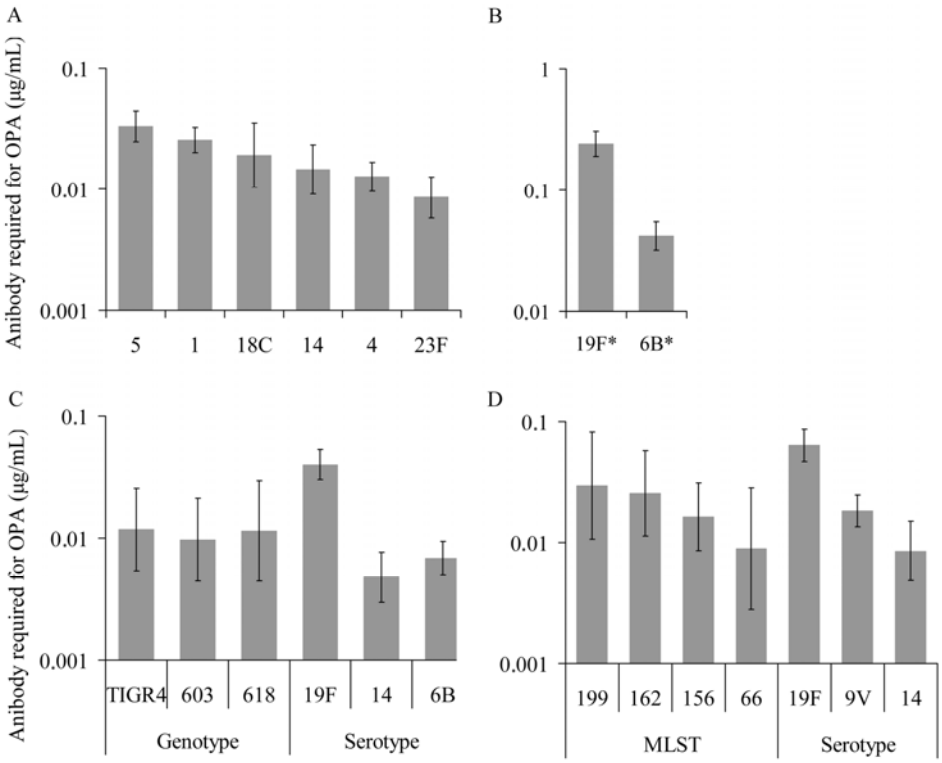


Figure 6. Opsonophagocytic killing of pneumococci. The sensitivity of pneumococcal isolates to opsonophagocytic killing was assessed by allowing human HL-60 granlulocytic cells to phagocytose pneumococci after pre-opsonization of the bacteria in the presence of complement and sera from humans immunized with a pneumococcal conjugate vaccine. Clinical isolates (A, B), strains which share the same genetic background (genotype) and express different capsules (C) and clinical isolates which are clonally related (have the same MLST) but express different capsules (D) were compared. Opsonophagocytosis is expressed as the serotype-specific geometric mean antibody concentration required for 50% opsonophagocytic killing; 95% confidence intervals are given. Serotypes marked with an asterisk (in panel B) were analyzed in a different study than the other clinical isolates and the results of the opsonophagocytic killing assay are not directly comparable across the two studies. The data originates from Fig. 2A in III (panel A), Fig 1B in II (panel B) and Fig. 4B and 4C in IV (panels C and D, respectively).

Data from clinical trials demonstrate a correlation of serotype-specific vaccine efficacy with opsonophagocytic activity of the antibodies (98, 329). In our studies serotype 1 and 19F isolates required high concentrations of capsule-specific antibodies for opsonophagocytosis, which is in concordance with their less optimal vaccine efficacies. However, in The Gambia the conjugate vaccine offered a good protection against invasive pneumococcal disease caused by serotype 5 (321), yet in

our study it required the highest concentration of capsule antibodies for killing in OPA. It has been proposed that the minimum antibody concentration after the primary immunization series associated with long term protection against invasive disease would be in the range of 0.15 to 0.5 μg/mL (33). A meta-analysis of three clinical trials derived a protective concentration of 0.35 μg/mL for anticapsular antibodies to the serotypes of a licensed 7-valent pneumococcal vaccine (174). This concentration has been recommended by a WHO Working Group as applicable on a global basis for assessing the efficacy of future pneumococcal conjugate vaccines. In this thesis several clinical isolates were analyzed side by side with the reference strains used in the OPA assay, which confirmed the previous finding that up to 5 times higher geometric mean antibody concentration is required for opsonophagocytic killing of serotype 19F than 6B pneumococci (98, 329, 394). Serotype 1 and 5 clinical isolates were even more resistant to opsonophagocytic killing than those of serotype 19F. In line with the findings of this study, the geometric mean OPA titers of children immunized with the 13-valent pneumococcal vaccine were lower for serotypes 1 and 5 compared to the serotypes, other than 19F, of the 7-valent vaccine (59, 104, 185, 395). This data strongly suggests that a higher concentration of capsule antibodies may be required for protection from disease caused by some serotypes. The different susceptibilities of different serotypes to opsonophagocytosis and antibody-mediated protection should be taken into account when guidelines for serological correlates for vaccine efficacy evaluations are made.

5.2.2 The capsule affects complement resistance (II-IV)

In the complement assay deposition of C3 on the bacteria was studied by using intact human sera as the source of complement. In the initial analyses I compared C3 deposition on serotype 6B and 19F isolates allowing the activation of complement to occur for 1 and 5 minutes. Already after 1 minute incubation more C3 was bound on serotype 6B than 19F isolates. The difference increased after 5 minutes of incubation (Fig. 7), by which time C3 deposition had already reached a plateau. It was observed that complement activation was, predictably, a rapid process and prolonging the incubation time up to 30 minutes did not markedly enhance C3 deposition or alter the result. Therefore, in the comparison of pneumococcal serotypes, bacteria were incubated with the serum for 5 minutes.

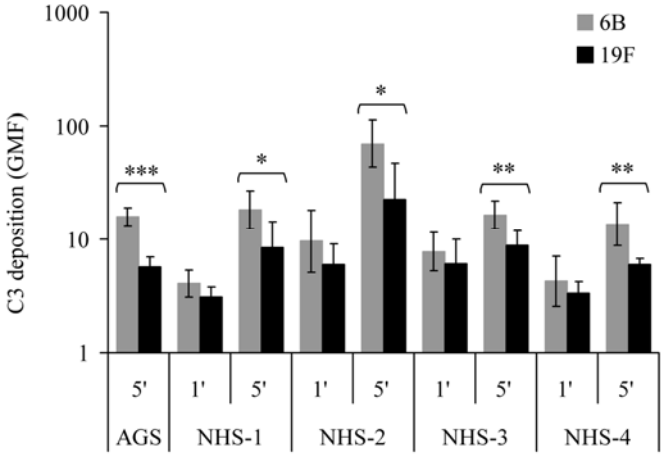


Figure 7. **C3 deposition on clinical isolates of serotypes 6B and 19F**. Bacteria were incubated with an agammaglobulinemic serum (AGS) and four different normal human sera (NHS) for 1 or 5 minutes. Deposition of C3 (C3b and iC3b) on the bacterial surface was detected with a fluorescently labeled anti-C3 antibody and measured by flow cytometry. Differences between serotypes were statistically significant after 5 minutes of incubation. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Student's t-test. Data originates from Fig. 4 of II. The results after 1 minute of incubation have not been previously published.

The comparison of several other clinical isolates revealed significant differences between serotypes in resistance to C3 deposition (Fig. 8a). Serotype 6B was more sensitive and serotype 1 more resistant to C3 deposition than any of the other serotypes. The clinical isolates within serotypes 1, 14, 23F, and 18C varied significantly in sensitivity to complement, indicating the significance of serotype independent features of the isolates in their resistance to complement. It was found that the isolates expressing different capsular serotypes differed significantly in their resistance to complement deposition, but differences between genotypes or MLSTs were much smaller than the differences between serotypes (Fig. 8b, c). In combination with any genetic background serotype 19F was always the most resistant capsule type. The concentration of anti-capsular antibodies present in the NHS was higher against 19F than against most other serotypes (Table 2), indicating that a low concentration of antibodies against 19F polysaccharide does not explain the complement resistance of serotype 19F isolates.

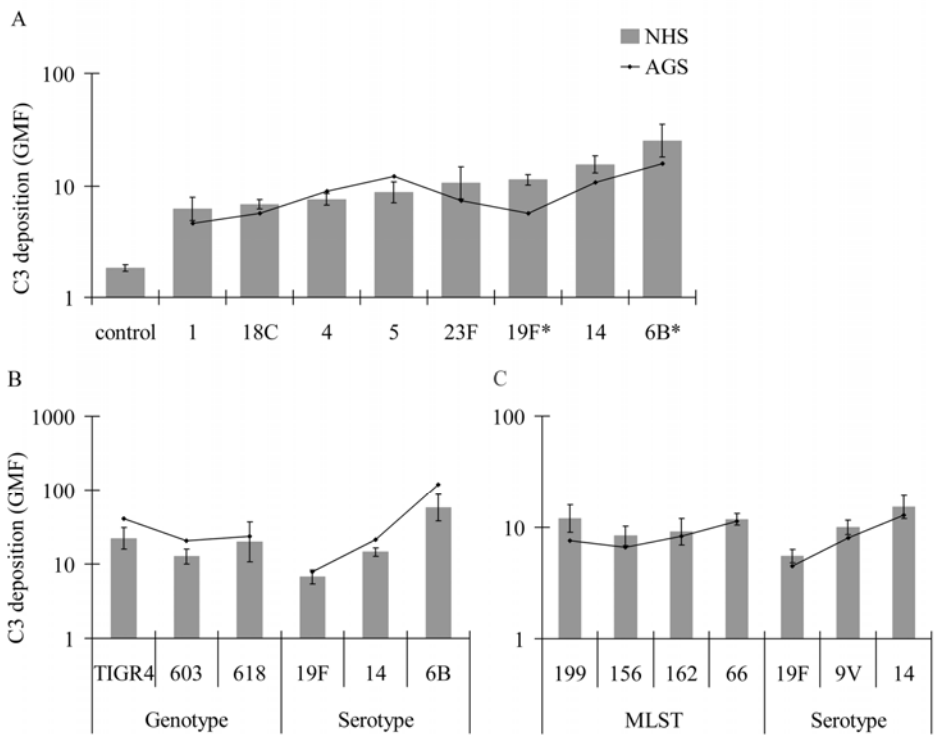


Figure 8. C3 deposition on pneumococci was measured by incubating the bacteria in 20% serum, after which deposition of C3 (C3b and iC3b) on the bacterial surface was detected with fluorescently labeled anti-C3 antibody and measured by flow cytometry. Clinical isolates (A), strains which share the same genetic background (genotype) and express different capsules (B) and clinical isolates, which are clonally related (have the same MLST) but express different capsules (C) were compared. Serotypes marked with an asterisk were analyzed in a different study than the other serotypes in panel A. Geometric mean intensities of fluorescence (GMF) are shown with 95% CI. NHS, normal human sera; AGS, agammaglobulinemic serum. Each isolate was analysed with ten different NHS and once with the AGS. AGS results have not been previously published. Data presented in panel A originates from Fig. 1A of III and data in panels B and C from Fig. 2B and 2C of IV.

The pneumococcal teichoic acid, also known as C-polysaccharide (C-PS), is a cell wall component common to all pneumococci, which binds serum C-reactive protein (CRP) (390). CRP has been shown to bind to several strains of *S. pneumoniae* including serotypes 3, 4, 6, 14, 19, 23 and 27, and the unencapsulated strain R36A (244). The opaque phenotype expresses less C-PS compared with the transparent form (379). CRP bound to pneumococci binds to C1q and activates the classical pathway of complement (313). CRP-mediated complement activation is restricted to the early part of the pathway, which generates opsonins that target the CRP-bound substances for opsonophagocytosis (28). Surface bound CRP reduces deposition of

C3b as a result of recruitment of factor H (171, 243) and C4BP which competes with C1q for binding to CRP (340). The ability of CRP to recruit factor H during complement activation has been suggested to support opsonic activation of complement without contributing further to the inflammatory state (244). For the analyses of this thesis, the bacteria were cultured in liquid medium, which has been shown to favor opaque colony morphology (266). The level of C-PS expression by the pneumococcal strains was not measured, but since they were all cultured with the same method, differential expression of C-PS by different serotypes, due to a different phase, is unlikely to explain the differences observed between the serotypes in resistance to C3 deposition. Moreover, the differences in C3 deposition between the serotypes were observed also between capsule-switch mutants that share otherwise the same genetic background. CRP is an acute phase serum protein, which only increases dramatically in concentration during inflammation. In healthy young adult volunteer blood donors, the median concentration of CRP is 0.8 µg/mL but during inflammation or tissue injury it may rise up to 500 µg/mL (70, 282). The sera used in the C3 deposition assay were collected from healthy young adults. The concentration of CRP in the sera was not measured, but because each strain in the same study was analyzed with the same set of sera, the CRP level in the sera should not affect the ultimate result.

Significant correlations were found between complement deposition and serum antibody concentration to pneumococcal surface proteins (PspA, PspC, and PhtD) in the normal human sera, whereas antibody concentrations to only a few capsule serotypes correlated with C3 deposition. Hierarchies of complement activation have indicated that IgG1 along with IgG3 is the most potent subclasses at mediating activation of the complement cascade, while IgG2 is a very poor activator (62). IgG2 is the dominating IgG subtype in response to pneumococcal capsular polysaccharides (24). Complement deposition was increased when the complement source was serum from an individual immunized with a conjugate vaccine. This suggested that C3 deposition was also dependent on the concentration of anti-capsular antibodies. Immunization with a conjugate vaccine has been reported to alter the ratio of IgG2 to IgG1 by inducing a higher proportion of IgG1 (347). The percentage of bacteria positive for C3 binding was similar with 6B and 19F when the bacteria were incubated with immune serum containing a three-fold antibody concentration to 19F compared to 6B. However, even though antibodies to capsular polysaccharides clearly enhanced C3 deposition, they did not even out the difference in C3 deposition between the two serotypes, when the geometric mean intensities of fluorescence were compared. The polarization of serotypes in susceptibility to C3 deposition was evident also when the source of complement was an agammaglobulinemic serum (Fig. 8).

The early studies of Hostetter, performed by immunoblotting, suggested that the ratio of the iC3b to C3d (both degradation fragments of C3b) rather than the total amount of C3 deposited on pneumococci would explain differences between serotypes in resistance to opsonophagocytosis, because the most important complement receptor, CR3, recognizes iC3b, but not C3d (156). At the beginning of this study, I measured the relationship of C3b binding and its degradation to iC3b by EIA and visualized the degradation of C3 fragments on serotype 6B and 19F isolates by immunoblotting using methods described previously (170). The very first (unpublished) results suggested that serotype 19F strains could degrade C3 more efficiently, but as the analyses were repeated, it was found that the reproducibility of the assays was not good. In flow cytometric analysis of C3 deposition, a clear association was found between the total amount of C3 detected on the bacterial surface and sensitivity to opsonophagocytosis, which is in contrast to Hostetter's hypothesis. Several bacterial pathogens have been shown to favor the inactivation of C3b to iC3b, e.g. by sialylation of capsular polysaccharides and by employment of factor H on the bacterial surface (201). Rapid cleavage of C3b to iC3b is favorable to the pathogen, as it prevents formation of new convertases and thereby inhibits deposition of new C3b molecules on the bacterial surface. Rapid breakdown of C3b to iC3b, thus, results in a decreased net C3 deposition.

There was no difference in complement deposition between mucosal and invasive isolates of the same serotype. In contrast to our finding, Sabharwal et al. (2009) observed that serotype 6A strains isolated from invasive infections were less susceptible to C3 deposition than strains of the same serotype isolated from the nasopharynx (322). Factor H binding by pneumococci is mostly mediated by PspC (402). Quin et al. (2006) reported that carriage isolates recruited significantly more factor H to their surfaces than either systemic or mucosal isolates. This binding was independent of the capsular serotype (291). Yuste et al. (2010) found that there was no significant correlation between the degree of factor H binding to pneumococcal strains and the effects of loss of PspC on C3 deposition (402). This suggested that there is no obvious association between the quantity of factor H on the bacterial surface and effects on complement deposition. In this study, all serotype 6B and 19F clinical isolates bound factor H, but there was no correlation between C3 deposition and factor H binding. This is in line with the previous studies and indicates that the differences in the efficiency of factor H binding could not account for the serotype-related differences seen in complement resistance. The differences observed in C3 deposition between serotypes, but not between genotypes, most likely result from the polysaccharide capsule, which is the most important factor contributing to the complement resistance of pneumococci.

Hyams et al. (2010) reported large differences in C3 deposition on opaque-phase variants of TIGR4 expressing serotype 4, 6A, 7F, and 23F capsules, even though the thicknesses of the capsule layers were similar (163). Timely regulation of capsule expression could be more important than its thickness to the ability of pneumococci to colonize and cause invasive disease. At the early stages of colonization, the capsule inhibits trapping of the pathogen to the luminal mucus and clearance by mucociliary flow (258). On the epithelial side of the mucus, a reduced amount of capsule promotes colonization by exposing adhesins and strengthening intimate contact with the epithelial cells (135). Once invasive disease is established, heavily encapsulated bacteria are better protected from phagocytes (189). It is believed that the net charge of each serotype, as well as the chemical composition, is the central reason for their virulence. Most capsule serotypes are highly charged at physiological pH, and this can directly interfere with interactions with phagocytes (207). To explain the differences observed between serotypes in resistance to C3 deposition, I looked for potential differences in the number of available targets for C3 deposition in the capsular polysaccharides, based on their published chemical compositions (191). Activated C3 forms a covalent bond with hydroxyl and amino groups on biological targets, via an acyl group. A significant association was found between the numbers of hydroxyl groups per polysaccharide repeat unit and complement deposition on the serotype: a high number of free hydroxyl groups was associated with increased C3 deposition (Fig. 5B of III). It was found that the serotypes 1 and 5, which were the most resistant to C3 opsonization, have a smaller number of targets (-OH or $-NH_2$ groups) available for C3b to make ester or amide linkages than for example serotypes 6B and 23F (examples shown in Fig. 9). These groups are also more often acetylated on serotypes resistant to C3 deposition than the targets on serotypes which are less resistant to C3 deposition. Thus, a low number of targets available for complement deposition and the presence of acetyl groups in the amino and carboxyl groups of the sugars of the capsule polysaccharide seem to be advantageous to resisting recognition by innate immune mechanisms.

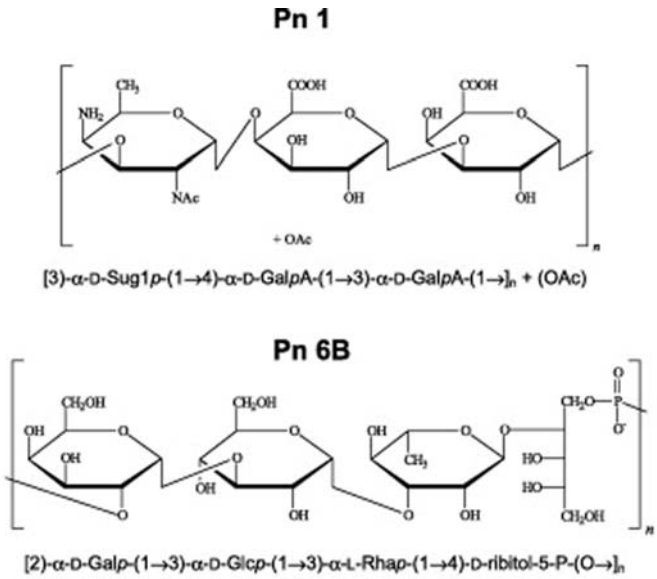


Figure 9. Repeating unit structures of the serotype 1 and 6B pneumococcal polysaccharides. Sug1, 2-acetamido-4-amino-2,4,6-trideoxy-d-galactose; Gal, galactose; Glc, glucose; Rha, rhamnose. Reproduced from (191) with the permission of the copyright holder, Elsevier Inc. In the repeating sugar unit of serotype 1 there are 6 hydroxyls, of which three are in acidic carboxyls, and one of the two amino groups is acetylated. In comparison, serotype 6B has 11 hydroxyls, none of them in carboxylic acids and there is no acetylation.

5.2.3 Resistance to complement deposition is associated with decreased susceptibility to opsonophagocytic killing (II-IV)

It is well-known that neutrophil phagocytosis is markedly increased by opsonization of pneumococcus with complement (404). In a study by Hyams et al. (2010), complement deposition on isogenic TIGR4 mutants expressing different serotypes correlated with neutrophil phagocytosis in the presence of complement, but differences in phagocytosis between strains were abolished when the bacteria were not opsonized (163). Antibodies activate the classical pathway of complement and, thus, enhance opsonophagocytosis. On the other hand, the importance of phagocytosis mediated by Fc receptors seems to be less important for protection against pneumococcal infection (323). Killing in the opsonophagocytic assay, applied in the studies of this thesis, is strongly mediated by complement and complement receptors. The polymorphonuclear HL-60 cells used in the assay express complement receptors CR1 and CR3 (for C3b and iC3b) and a low-affinity receptor Fc RII for IgG (308).

It was found that the pneumococcal serotypes which required a higher concentration of anti-capsular antibody for opsonophagocytic killing, were also among the serotypes least susceptible to opsonization with C3b (Fig. 10). Serotype 1 was especially resistant to complement, which could be one explanation for the poor efficacy of conjugate vaccines to invasive disease caused by this serotype (79, 321). In contrast, serotype 6B was consistently the most susceptible serotype to C3 deposition. This might explain why the conjugate vaccine was found to be efficacious in protection against AOM caused by 6B at a relatively low antibody concentration (176). Differences between serotypes in sensitivity to opsonophagocytosis could result not only from the amount of complement deposited, but also from the type of exposure of complement to the phagocytes due to the different capsular structures and localization of the opsonins.

The difference in sensitivity to complement deposition is not likely to be the only factor explaining the different susceptibilities of the serotypes to opsonophagocytic killing. Despite moderate resistance to complement deposition, a very high concentration of serotype-specific antibodies was found to be required for opsonophagocytosis of serotype 19F strains, suggesting inferior functional properties of antibodies. In a previous study, the correlation of antibody concentration and avidity was compared with opsonophagocytic activity, and it was found that the concentration of specific antibodies to serotype 6B and 19F polysaccharides is distinctly the most important factor contributing to opsonophagocytic killing, whereas no significant contribution of antibody avidity was found (10). The results nevertheless suggested a statistically non-significant trend that less antibodies of high avidity than low avidity were needed for killing bacteria. In later studies, no such association could be demonstrated (98, 393). It is possible that high-avidity antibodies more strongly enhance complement deposition than weak-avidity antibodies. The serum antibody concentration affects both opsonophagocytic killing and complement deposition. These two measures seem to be linked, but the role of antibody avidity in the process remains unclear.

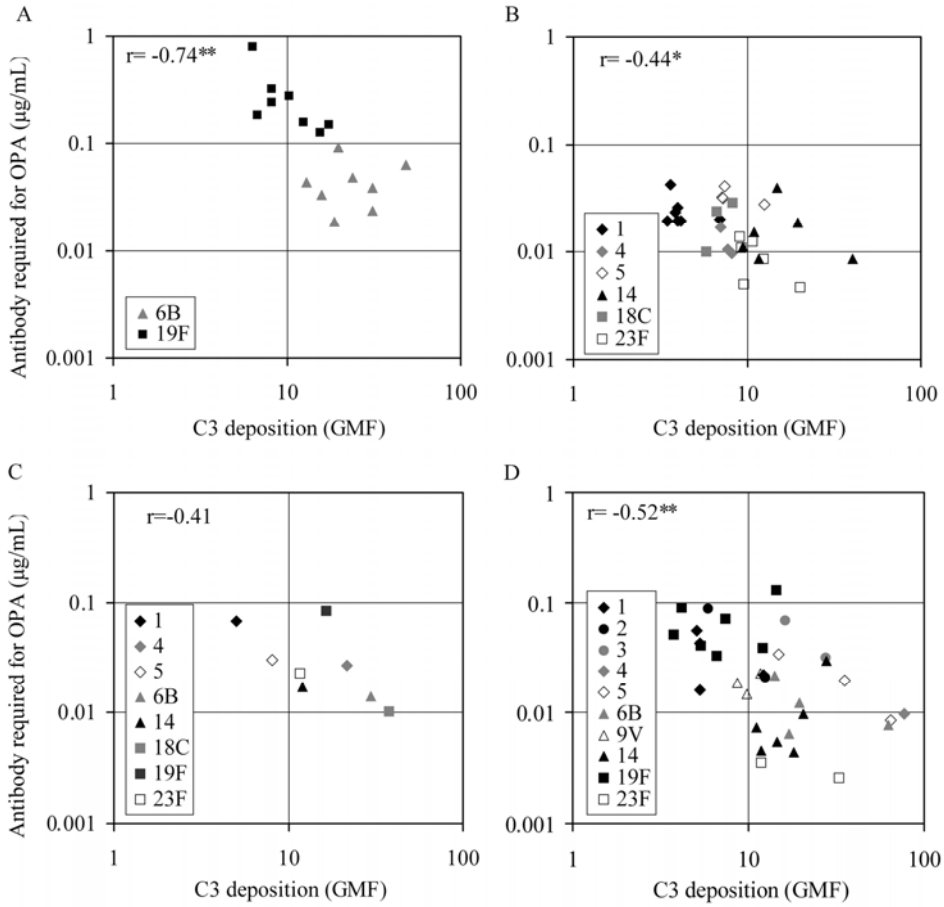


Figure 10. **Correlation between complement deposition and OPA killing**. Pneumococcal isolates representing different capsular serotypes and genotypes were compared; clinical isolates (A, B), pneumococcal reference strains (C), and strains that expressed different capsular serotypes in the same genotype and the capsule donor strains (D). Geometric mean intensities of fluorescence (GMF) corresponding to C3 deposition and geometric mean anti-capsular antibody concentrations required for 50% opsonophagocytic killing of pneumococci are shown. The serotypes, which were the most resistant to C3 deposition, required the highest concentration of serotype-specific capsule antibodies for opsonophagocytic killing. Statistically significant correlations are marked with an asterisk (Pearson's correlation coefficient), *$p<0.05$, **$p<0.01$. Data in panels B and C originates from Fig. 3A and 3B from III. Previously unpulished data is presented in panels A and D.

5.3 Resistance to complement and opsonophagocytic killing is associated with serotype-specific mortality in invasive pneumococcal disease (III-IV)

Considering the extent of exposure to pneumococci, invasive disease is rare and a number of virulence factors influence the success of pneumococcal isolates to transit from nasopharyngeal carriage to causing invasive disease (144). The capsular serotype is assumed to be more important than the genotype for the ability of pneumococcus to cause invasive disease (54, 56, 137). The invasive disease potential is a measure of the ability of pneumococci to progress from nasopharyngeal carriage to invasive disease. It is estimated by comparing nasopharyngeal carriage rates of pneumococcal serotypes (or strains) with their rates in invasive pneumococcal disease (IPD) (54). This is similar to the attack rate, which is the risk of disease as a result of pathogen acquisition (54, 342). Certain serotypes, such as 1, 4, 5, 7F, and 14, appear to be associated with invasive disease, whereas some serotypes, such as 6B, 19F and 23F, have a low invasive disease potential although they also occasionally cause invasive disease (54, 56, 137, 200, 320, 332, 342).

Invasiveness of pneumococcal serotypes is not equivalent to their virulence (mortality). Mortality following IPD caused by different serotypes was assessed in a large population-based study by Harboe et al. (2009), by using a multivariate logistic regression analysis, in which odds-ratio estimates were calculated for the most frequent serotypes (138). A case was defined as an occurrence of IPD confirmed by positive culture for *S. pneumoniae* from a hospitalized patient's cerebrospinal fluid or blood. The OR estimates were adjusted for age, sex, time at diagnosis, IPD focus (bacteremia or meningitis), alcoholism-related conditions and low, medium or high comorbidity score and ORs were calculated for serotypes ≥ 50 IPD cases only (138). In patients older than 5 years, certain serotypes, e.g. 3 and 19F, were found to have a highly increased mortality as compared with serotype 1. In children younger than 5 years of age, serotypes 14, 6A, 7F, and 4 tended to be associated with a *decreased* mortality.

The higher mortality noted with opportunistic serotypes might be determined by host rather than by pathogen-derived factors. Pneumococcus tends to infect patients with an impaired immune system, and this behavior seems to be even more pronounced for opportunistic serotypes. Previous studies have indicated that highly invasive serotypes, such as 1, 7F and 14, are associated with lower mortality proportions and are more likely to be invasive in younger patients who have fewer comorbid conditions (341). In another meta-analysis of serotype-specific disease outcomes, significant differences were found in the risk ratio estimates among

serotypes in patients with bacteremic pneumonia (377). Weinberger et al. (2010) concluded that the serotypes with an increased risk of death (3, 6A, 6B, 9N, and 19F) had high carriage prevalence and low invasiveness (377), whereas serotypes 1, 7F, and 8 were associated with a decreased risk of death (377). It has been suggested that capsular serotypes, which are carried for a short duration and have a high attack rate, such as serotypes 1, 5, and 7F, which affect previously healthy individuals and are associated with lower mortality, behave like "primary pathogens" (342). Meanwhile, serotypes that are carried for a long duration, and which have a low invasive disease potential, such as 6B, 19F, and 23F, behave like opportunistic pathogens, causing disease in patients with an underlying condition and are associated with a more severe disease and higher mortality (138, 341).

In this thesis, I found a serotype-related relationship between the severity of IPD, described by Harboe et al. (2009) (138), and resistance to C3 deposition and opsonophagocytic killing (Fig. 11). This suggests that the primary pathogens, which are associated with a lower mortality than the opportunistic serotypes, are more resistant to C3 deposition and require a higher concentration of capsule antibodies for opsonophagocytic killing. Despite the importance of capsular serotype as a determinant of invasiveness, clinical pneumococcal isolates belonging to different MLSTs have been found to have different invasive disease potentials (137). It was possible to detect a trend for serotype-specific correlation between IPD mortality and resistance to complement and opsonophagocytosis not only with clinical isolates, but also with capsule-switched mutants and strains that share the same MLST and express different capsules (Fig. 11). Serotypes 3 and 19F are relatively resistant to complement and require an exceptionally high concentration of antibodies for killing, yet their IPD mortalities are notably high. If these two serotypes were excluded from the statistical analysis, the correlations reached statistical significance (Fig. 11). Because the complement system and phagocytosis have central roles in systemic immunity to *S. pneumoniae*, the differences observed between pneumococcal serotypes in the ability to cause invasive disease may reflect the relative capacity of the serotypes to activate innate immune responses. The correlation of serotype-specific complement resistance with IPD severity may indicate that primary pathogens, which are able to cause disease in healthy individuals and have a high potential to cause invasive disease (54, 56, 327, 341), are better able to resist host immunity than the opportunistic serotypes.

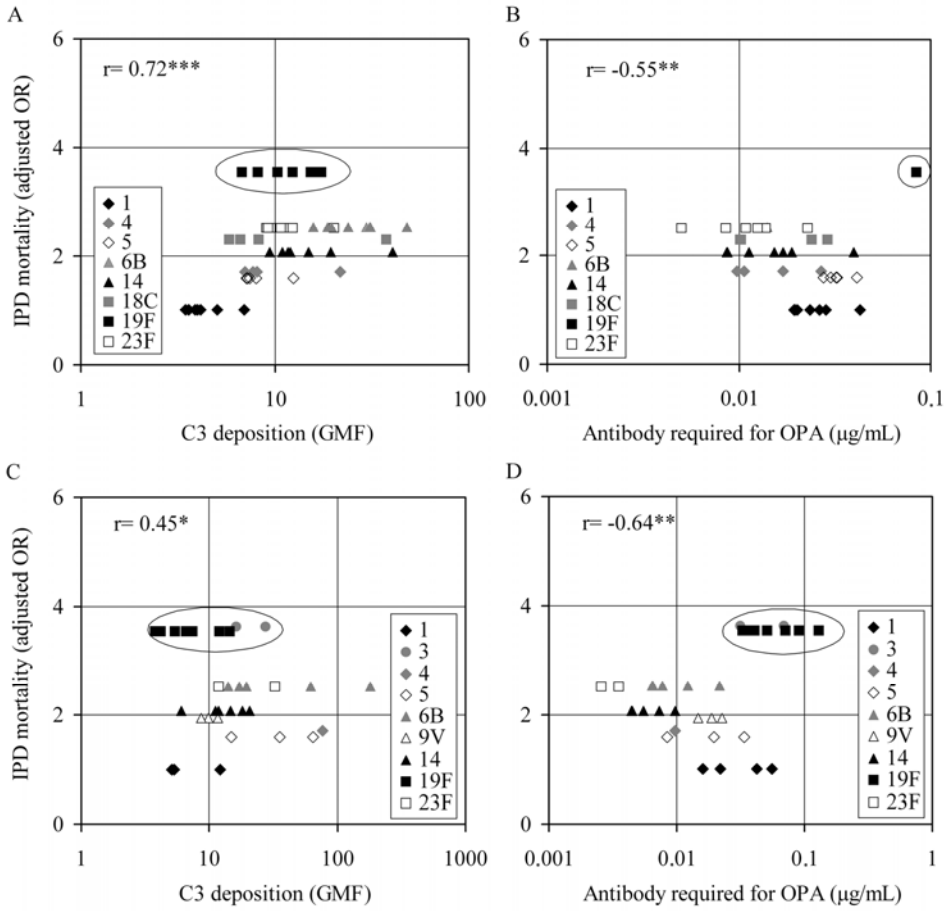


Figure 11. Correlation between serotype-specific mortality to invasive pneumococcal disease (IPD), C3 deposition, and OPA killing. Pneumococcal isolates representing different serotypes and genotypes were compared; clinical isolates and reference strains (A, B) and isogenic strains expressing different capsules in the same genotype and the capsule donor strains (C, D). Serotype-specific mortality to IPD adjusted for age, comorbidity and other factors as described by Harboe et al. (138) is shown on the y-axis. Geometric mean intensities of fluorescence (GMF) corresponding to C3 deposition and geometric mean anti-capsular antibody concentrations required for 50% opsonophagocytic killing are shown on the x-axis. Serotypes 3 and 19F (circled) differ from the others by being intermediate in resistance to complement yet requiring a high antibody concentration for opsonophagocytosis (see Fig. 10). These serotypes were excluded from the statistical analysis. Correlation was weaker in panels A (0.48**) and B (-0.25, non-significant) if serotype 19F was included. In panels C and D the correlations were no longer seen if serotypes 3 and 19F were included. *p<0.05, **p<0.01, ***p<0.001, Pearson's correlation coefficient. Data in panels A and B originate from Fig. 4A and 4B in III. Previously unpublished data is presented in panels C and D.

Weinberger et al. described an association between serotype prevalence in carriage and the amount of capsular polysaccharide produced *in vitro* (378). The serotypes with less complex polysaccharides were suggested to be associated with a higher prevalence in carriage (378). The serotypes more prevalent in carriage were found to have a higher risk ratio in bacteremic pneumonia and be more heavily encapsulated *in vitro* (377). A significant association was found between the number of carbons in the polysaccharide repeat unit and the severity of IPD caused by different pneumococcal serotypes. The serotypes which have the highest IPD mortality and which are also the most prevalent in carriage, tend to have the most energy-efficient structure (least carbons per polysaccharide repeat unit). The strong negative correlation between the severity of IPD and metabolic cost of capsular polysaccharide unit synthesis suggests that the serotypes which have a metabolically costly capsule, cause less severe disease. However, these serotypes are also the ones that are highly invasive.

Disease represents a dead end for the organism, especially when the result is the rapid demise of the host (253). In contrast, transmission occurs from the reservoir of pneumococci residing asymptomatically in the nasopharynx (16). Why has pneumococcus evolved virulent, invasive behavior of certain capsular polysaccharide coats? Modeling of interspecies interaction between *S. pneumoniae* and *Haemophilus influenzae* during experimental colonization indicated that the more virulent pneumococcal serotype is able to outcompete the other species and survive, even if production of a capsule is otherwise costly (224). Pneumococci expressing the avirulent capsule were sensitive to the effects of complement and neutrophils, whereas strains expressing the virulent capsule type were not killed (224). The capsule type may have a profound effect on not only survival in invasive disease, but also on the outcome of mucosal competition by allowing for persistence during mucosal inflammation induced by competitive interactions among the nasopharyngeal flora.

6 CONCLUSIONS

Opsonization of *S. pneumoniae* with complement (C3b and iC3b) is a major mechanism of immune protection against invasive pneumococcal disease. Like many pathogenic bacteria, pneumococcus has several tools to counteract innate immunity. Immunization with conjugate vaccines produces opsonic antibodies, which enhance complement deposition and clearance of the bacteria. Data from clinical studies suggest that the ability of these vaccines to protect against pneumococcal disease depends on the serotype. The genotype of the pneumococcal strain can also have a significant influence on its virulence both *in vivo* and *in vitro*. In the work presented in this thesis, I have assessed the relative roles of different pneumococcal virulence factors in complement resistance of the species.

The capsular polysaccharide is a major pneumococcal virulence factor that triggers a specific immunological response to infection and vaccination. It constitutes an important determinant of the potential of pneumococcus to cause invasive and severe disease. I found that pneumococcal isolates have different susceptibilities to complement deposition and antibody-mediated phagocytosis. The genotype of the strain affects its resistance to complement, which was seen as variation between strains sharing the same capsule type. However, the results of the studies of this thesis studies suggest that the polysaccharide capsule is more important than the genotype in determining the resistance of the strain to complement and opsonophagocytosis. A higher concentration of anti-capsular antibodies was required for the opsonophagocytic killing of the serotypes which were most resistant to complement. Serotype-related resistance to complement offers an explanation for the differences observed in the efficacy of conjugate vaccines against pneumococcal serotypes. Differences in the chemical composition of the capsular polysaccharides could partly explain the differences in complement deposition observed between serotypes. A low number of available targets for C3b deposition (hydroxyl groups) and the presence of acetyl groups in the amino and carboxyl groups of the sugars of the capsule polysaccharide appear to be advantageous to resisting recognition by the complement system and phagocytic cells.

Differences between serotypes in their ability to activate innate immune responses may contribute to disease severity as well as bacterial clearance. The serotypes that are able to persist in the respiratory mucosa of healthy individuals are crucial for the survival and spread of the bacterium. The prevalence of a serotype in nasopharyngeal carriage seems to be determined by the metabolic cost the bacteria invest in a capsule. The serotypes associated with a higher prevalence in carriage tend to have an energy-efficient structure (less carbons per polysaccharide repeat

unit), whereas the capsular polysaccharides of invasive serotypes are more complex. The invasive serotypes in general appear to be more resistant to complement and opsonophagocytic killing than the opportunistic serotypes. However, invasive pneumococcal disease caused by invasive serotypes is associated with a less severe outcome. It is possible that the capsule affects not only survival of the bacterium in the circulation, but also success in colonization. The latter allows bacterial persistence during mucosal inflammation induced by competitive interactions among the local microflora.

Although the capsule is significant in many aspects of pneumococcal pathogenesis, the virulence proteins are also essential, as demonstrated by the loss of virulence in mutant strains. Protection of pneumococci against complement deposition involves the coordinated efforts of many different surface proteins. I found that Pht proteins may have a role in complement evasion, but their significance depends on the genotype of the strain. Other virulence proteins involved in inhibition of complement deposition, such as PspA, PspC, and pneumolysin, may compensate for the lack of Phts. The main virulence mechanism of Phts could also be other than complement inhibition. Immunization with a mixture of these proteins has been shown to enhance the survival of mice from pneumococcal carriage or disease in previous studies. A combination of pneumococcal protein antigens common to all serotypes in a pneumococcal vaccine could provide non-serotype-dependent protection.

Both the capsular serotype and the genotype contribute to the virulence of pneumococcal strains. In virulent strains, successful combinations of the capsule and virulence proteins may have co-evolved into optimal sets of genes. In these combinations, the capsule may play the major role – but the same set of proteins may act differently with various capsular types. Since certain capsule types endow pneumococci with more resistance to complement deposition than others, it is apparent that more anti-capsular antibodies are required for efficient opsonophagocytosis of the more resistant serotypes. The different susceptibilities of different serotypes to complement deposition, opsonophagocytosis, and resultant antibody-mediated protection should be taken into account when guidelines for serological correlates for vaccine efficacy evaluations are made.

7 ACKNOWLEDGMENTS

This study was conducted at the National Institute for Health and Welfare (THL), Department of Vaccination and Immune Protection, Immune Response Unit and at the Haartman Institute, Department of Bacteriology and Immunology, University of Helsinki. This work received financial support from GlaxoSmithKline Biologicals. I acknowledge the Director General of THL, Pekka Puska, the former Head of the National Public Health Institute (KTL), Jussi Huttunen, and the Head of the Department, Terhi Kilpi, for providing excellent research facilities. I sincerely thank Research Professor Outi Vaarala, the head of the Immune Response Unit, for guiding the laboratory through new challenges and for reminding me that the work we do is significant.

This thesis was jointly supervised by Research Professor Helena Käyhty, Professor Seppo Meri, and Docent Merja Väkeväinen. I express my gratitude to them for their valuable advice, support, and guidance over the years. I wish to thank Helena Käyhty for introducing me to the field of vaccine immunology and for giving me the opportunity to work on this thesis. I appreciate her excellent knowledge and humble mind, and I thank her for believing in me from the beginning. I am equally grateful for Seppo Meri for sharing his vast knowledge on the complement system and for his enthusiastic attitude and encouragement. I am thankful that I had the opportunity to spend time in his laboratory, where the work of this thesis was begun. I wish to express my warmest thanks to Merja Väkeväinen for her wholehearted support and guidance during the work of my thesis and also for the pleasant times shared.

I thank the co-authors of the original publications for their excellent collaboration. I owe my special tanks to Dr. Krzysztof Trzciński, my co-author in two articles, for investing time on the manuscripts and sharing his novel ideas. I thank Dr. Jan Poolman for his support and insightful scientific advice in the study design. I am grateful for Academician Pirjo Mäkelä and Professor David Briles from the University of Alabama at Birmingham, Alabama, for volunteering to comment on the manuscripts. I thank Professor Marc Lipsitch from the Harvard School of Public Health, Boston, MA, for discussing the work and for his valuable scientific advice.

I sincerely thank all the collaborators, who were kind enough to share their material and ideas, which made the studies of this thesis possible. I am especially indebted to Dr. Hanna Jarva who taught me several complement assays in Seppo Meri's laboratory. Her enthusiastic and optimistic attitude is a pleasure to experience. I also thank Dr. Tarja Kaijalainen from THL, Oulu, Finland, for supplying the clinical pneumococcal isolates collected from Finnish patients and Dr. William P. Hanage

from Imperial College, London, United Kingdom, for agreeing to share the MLST data of the pneumococcal strains. I want to thank statistician Mika Lahdenkari for patiently advising me on my many questions on the statistical analyses.

The reviewers, Dr. Sakari Jokiranta and Dr. Kaarina Lähteenmäki, are acknowledged for patiently investing their time and effort to provide constructive criticism towards this work, improving its quality.

It has been a pleasure to work with the present and past co-workers of the vaccine immunology laboratory. My special thanks are due to Leena Tikkanen, who assisted with the laboratory analyses in a meticulous manner and who was always pleasant company. I want to thank my colleagues Nina Ekström, Emma Holmlund, Dr. Anu Nurkka, Dr. Taneli Puumalainen, Dr. Birgit Simell, Dr. Anu Soininen, Dr. Maija Toropainen, and Arja Vuorela as well as the laboratory personnel of the vaccine immunology laboratory: Raili Haikala, Anne Holm, Maija Idman, Teija Jaakkola, Kaisa Jousimies, Hannele Lehtonen, Päivi Paalanen, Sanna Piipponen, Leena Saarinen, Camilla Virta, secretary Anna Lundqvist and IT support person Piia Korkeamäki for their pleasant company. I also thank my new colleagues at the Immune Response Unit for providing a welcoming, scientific atmosphere.

On a personal note, I would like to warmly thank my family, my parents, sister and grandmother, for their support and encouragement and my dear friends and those close to me for all the good times shared over the years.

Helsinki, April 2011

Merit Melin

8 REFERENCES

1. **Abeyta, M., G. G. Hardy, and J. Yother.** 2003. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. Infect Immun **71:**218-225.
2. **Adamou, J. E., J. H. Heinrichs, A. L. Erwin, W. Walsh, T. Gayle, M. Dormitzer, R. Dagan, Y. A. Brewah, P. Barren, R. Lathigra, S. Langermann, S. Koenig, and S. Johnson.** 2001. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. Infect Immun **69:**949-958.
3. **Adegbola, R. A., P. C. Hill, O. Secka, U. N. Ikumapayi, G. Lahai, B. M. Greenwood, and T. Corrah.** 2006. Serotype and antimicrobial susceptibility patterns of isolates of Streptococcus pneumoniae causing invasive disease in The Gambia 1996-2003. Trop Med Int Health **11:**1128-1135.
4. **Agarwal, V., T. M. Asmat, S. Luo, I. Jensch, P. F. Zipfel, and S. Hammerschmidt.** 2010. Complement regulator Factor H mediates a two-step uptake of Streptococcus pneumoniae by human cells. J Biol Chem **285:**23486-23495.
5. **Aguiar, S. I., I. Serrano, F. R. Pinto, J. Melo-Cristino, and M. Ramirez.** 2008. The presence of the pilus locus is a clonal property among pneumococcal invasive isolates. BMC Microbiol **8:**41.
6. **Allegrucci, M., F. Z. Hu, K. Shen, J. Hayes, G. D. Ehrlich, J. C. Post, and K. Sauer.** 2006. Phenotypic characterization of Streptococcus pneumoniae biofilm development. J Bacteriol **188:**2325-2335.
7. **Alonso De Velasco, E., A. F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: Virulence factrors, pathogenesis, and vaccines. Microbiological Reviews **59:**591-603.
8. **Anderton, J. M., G. Rajam, S. Romero-Steiner, S. Summer, A. P. Kowalczyk, G. M. Carlone, J. S. Sampson, and E. W. Ades.** 2007. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. Microb Pathog **42:**225-236.
9. **Angel, C. S., M. Ruzek, and M. K. Hostetter.** 1994. Degradation of C3 by Streptococcus pneumoniae. J Infect Dis **170:**600-608.
10. **Anttila, M., M. Voutilainen, V. Jantti, J. Eskola, and H. Kayhty.** 1999. Contribution of serotype-specific IgG concentration, IgG subclasses and relative antibody avidity to opsonophagocytic activity against Streptococcus pneumoniae. Clin Exp Immunol **118:**402-407.
11. **Arlaud, G. J., C. Gaboriaud, N. M. Thielens, and V. Rossi.** 2002. Structural biology of C1. Biochem Soc Trans **30:**1001-1006.

12. **Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik.** 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. J Exp Med **200:**267-272.
13. **Arredouani, M. S., Z. Yang, A. Imrich, Y. Ning, G. Qin, and L. Kobzik.** 2006. The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles. Am J Respir Cell Mol Biol **35:**474-478.
14. **Auranen, K., J. Mehtala, A. Tanskanen, and S. K. M.** 2009. Between-strain competition in acquisition and clearance of pneumococcal carriage--epidemiologic evidence from a longitudinal study of day-care children. Am J Epidemiol **171:**169-176.
15. **Austrian, R.** 1976. The quellung reaction, a neglected microbiologic technique. Mount Sinai Journal of Medicine **43:**699–709.
16. **Austrian, R.** 1986. Some aspects of the pneumococcal carrier state. J Antimicrob Chemother **18 Suppl A:**35-45.
17. **Austrian, R.** 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Rev Infect Dis **3 Suppl:**S1-17.
18. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. J. Exp. Med. **79 (1944):**137–158.
19. **Bagnoli, F., M. Moschioni, C. Donati, V. Dimitrovska, I. Ferlenghi, C. Facciotti, A. Muzzi, F. Giusti, C. Emolo, A. Sinisi, M. Hilleringmann, W. Pansegrau, S. Censini, R. Rappuoli, A. Covacci, V. Masignani, and M. A. Barocchi.** 2008. A second pilus type in Streptococcus pneumoniae is prevalent in emerging serotypes and mediates adhesion to host cells. J Bacteriol **190:**5480-5492.
20. **Balachandran, P., A. Brooks-Walter, A. Virolainen-Julkunen, S. K. Hollingshead, and D. E. Briles.** 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. Infect Immun **70:**2526-2534.
21. **Balachandran, P., S. K. Hollingshead, J. C. Paton, and D. E. Briles.** 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. J Bacteriol **183:**3108-3116.
22. **Baraff, L. J., S. I. Lee, and D. L. Schriger.** 1993. Outcomes of bacterial meningitis in children: a meta-analysis. Pediatr Infect Dis J **12:**389-394.
23. **Barocchi, M. A., J. Ries, X. Zogaj, C. Hemsley, B. Albiger, A. Kanth, S. Dahlberg, J. Fernebro, M. Moschioni, V. Masignani, K. Hultenby, A. R. Taddei, K. Beiter, F. Wartha, A. von Euler, A. Covacci, D. W. Holden, S. Normark, R. Rappuoli, and B. Henriques-Normark.** 2006. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A **103:**2857-2862.

24. **Barrett, D. J., and E. M. Ayoub.** 1986. IgG2 subclass restriction of antibody to pneumococcal polysaccharides. Clin Exp Immunol **63:**127-134.
25. **Basset, A., K. Trzcinski, C. Hermos, K. L. O'Brien, R. Reid, M. Santosham, A. J. McAdam, M. Lipsitch, and R. Malley.** 2007. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. J Clin Microbiol **45:**1684-1689.
26. **Beiter, K., F. Wartha, R. Hurwitz, S. Normark, A. Zychlinsky, and B. Henriques-Normark.** 2008. The capsule sensitizes Streptococcus pneumoniae to alpha-defensins human neutrophil proteins 1 to 3. Infect Immun **76:**3710-3716.
27. **Benton, K. A., J. C. Paton, and D. E. Briles.** 1997. The hemolytic and complement-activating properties of pneumolysin do not contribute individually to virulence in a pneumococcal bacteremia model. Microb Pathog **23:**201-209.
28. **Berman, S., H. Gewurz, and C. Mold.** 1986. Binding of C-reactive protein to nucleated cells leads to complement activation without cytolysis. J Immunol **136:**1354-1359.
29. **Berry, A. M., R. A. Lock, and J. C. Paton.** 1996. Cloning and characterization of nanB, a second Streptococcus pneumoniae neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli. J Bacteriol **178:**4854-4860.
30. **Berry, A. M., and J. C. Paton.** 2000. Additive attenuation of virulence of Streptococcus pneumoniae by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. Infect Immun **68:**133-140.
31. **Berry, A. M., and J. C. Paton.** 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae. Infect Immun **64:**5255-5262.
32. **Birmingham, D. J., and L. A. Hebert.** 2001. CR1 and CR1-like: the primate immune adherence receptors. Immunol Rev **180:**100-111.
33. **Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohberger, W. Watson, R. Austrian, and K. Edwards.** 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr Infect Dis J **19:**187-195.
34. **Blom, A. M., T. Hallstrom, and K. Riesbeck.** 2009. Complement evasion strategies of pathogens-acquisition of inhibitors and beyond. Mol Immunol **46:**2808-2817.
35. **Blom, A. M., L. Kask, and B. Dahlback.** 2003. CCP1-4 of the C4b-binding protein alpha-chain are required for factor I mediated cleavage of complement factor C3b. Mol Immunol **39:**547-556.
36. **Blom, A. M., B. O. Villoutreix, and B. Dahlback.** 2004. Functions of human complement inhibitor C4b-binding protein in relation to its structure. Arch Immunol Ther Exp (Warsz) **52:**83-95.

37. **Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen.** 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J Exp Med **188:**2381-2386.
38. **Bogaert, D., R. De Groot, and P. W. Hermans.** 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **4:**144-154.
39. **Bogaert, D., M. Sluijter, R. De Groot, and P. W. Hermans.** 2004. Multiplex opsonophagocytosis assay (MOPA): a useful tool for the monitoring of the 7-valent pneumococcal conjugate vaccine. Vaccine **22:**4014-4020.
40. **Bogaert, D., C. M. Thompson, K. Trzcinski, R. Malley, and M. Lipsitch.** 2010. The role of complement in innate and adaptive immunity to pneumococcal colonization and sepsis in a murine model. Vaccine **28:**681-685.
41. **Boulnois, G. J.** 1992. Pneumococcal proteins and the pathogenesis of disease caused by Streptococcus pneumoniae. J Gen Microbiol **138 (Pt 2):**249-259.
42. **Branger, J., S. Knapp, S. Weijer, J. C. Leemans, J. M. Pater, P. Speelman, S. Florquin, and T. van der Poll.** 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun **72:**788-794.
43. **Briles, D. E., E. Ades, J. C. Paton, J. S. Sampson, G. M. Carlone, R. C. Huebner, A. Virolainen, E. Swiatlo, and S. K. Hollingshead.** 2000. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of Streptococcus pneumoniae. Infect Immun **68:**796-800.
44. **Briles, D. E., S. Hollingshead, A. Brooks-Walter, G. S. Nabors, L. Ferguson, M. Schilling, S. Gravenstein, P. Braun, J. King, and A. Swift.** 2000. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. Vaccine **18:**1707-1711.
45. **Briles, D. E., S. K. Hollingshead, G. S. Nabors, J. C. Paton, and A. Brooks-Walter.** 2000. The potential for using protein vaccines to protect against otitis media caused by Streptococcus pneumoniae. Vaccine **19:**S87-95.
46. **Briles, D. E., S. K. Hollingshead, J. C. Paton, E. W. Ades, L. Novak, F. W. van Ginkel, and W. H. Benjamin, Jr.** 2003. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with Streptococcus pneumoniae. J Infect Dis **188:**339-348.
47. **Briles, D. E., S. K. Hollingshead, E. Swiatlo, A. Brooks-Walter, A. Szalai, A. Virolainen, L. S. McDaniel, K. A. Benton, P. White, K. Prellner, A. Hermansson, P. C. Aerts, H. Van Dijk, and M. J. Crain.** 1997. PspA and PspC: their potential for use as pneumococcal vaccines. Microb Drug Resist **3:**401-408.
48. **Briles, D. E., R. C. Tart, E. Swiatlo, J. P. Dillard, P. Smith, K. A. Benton, B. A. Ralph, A. Brooks-Walter, M. J. Crain, S. K. Hollingshead, and L. S. McDaniel.** 1998. Pneumococcal diversity: considerations for new vaccine

strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev **11:**645-657.

49. **Briles, D. E., J. Yother, and L. S. McDaniel.** 1988. Role of pneumococcal surface protein A in the virulence of Streptococcus pneumoniae. Rev Infect Dis **10 Suppl 2:**S372-374.
50. **Briles, E. B., and A. Tomasz.** 1973. Pneumococcal Forssman antigen. A choline-containing lipoteichoic acid. J Biol Chem **248:**6394-6397.
51. **Brock, S. C., P. A. McGraw, P. F. Wright, and J. E. Crowe, Jr.** 2002. The human polymeric immunoglobulin receptor facilitates invasion of epithelial cells by Streptococcus pneumoniae in a strain-specific and cell type-specific manner. Infect Immun **70:**5091-5095.
52. **Brooks-Walter, A., D. E. Briles, and S. K. Hollingshead.** 1999. The pspC gene of Streptococcus pneumoniae encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun **67:**6533-6542.
53. **Brown, J. S., T. Hussell, S. M. Gilliland, D. W. Holden, J. C. Paton, M. R. Ehrenstein, M. J. Walport, and M. Botto.** 2002. The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. Proc Natl Acad Sci U S A **99:**16969-16974.
54. **Brueggemann, A. B., D. T. Griffiths, E. Meats, T. Peto, D. W. Crook, and B. G. Spratt.** 2003. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. J Infect Dis **187:**1424-1432.
55. **Brueggemann, A. B., R. Pai, D. W. Crook, and B. Beall.** 2007. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog **3:**e168.
56. **Brueggemann, A. B., T. E. Peto, D. W. Crook, J. C. Butler, K. G. Kristinsson, and B. G. Spratt.** 2004. Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis **190:**1203-1211.
57. **Brugger, S. D., P. Frey, S. Aebi, J. Hinds, and K. Muhlemann.** 2010. Multiple colonization with S. pneumoniae before and after introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine. PLoS One **5:**e11638.
58. **Bruyn, G. A., B. J. Zegers, and R. van Furth.** 1992. Mechanisms of host defense against infection with Streptococcus pneumoniae. Clin Infect Dis **14:**251-262.
59. **Bryant, K. A., S. L. Block, S. A. Baker, W. C. Gruber, and D. A. Scott.** 2010. Safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine. Pediatrics **125:**866-875.
60. **Bunker, V. W., L. J. Hinks, M. S. Lawson, and B. E. Clayton.** 1984. Assessment of zinc and copper status of healthy elderly people using metabolic balance studies and measurement of leucocyte concentrations. Am J Clin Nutr **40:**1096-1102.

61. **Burman, L. A., R. Norrby, and B. Trollfors.** 1985. Invasive pneumococcal infections: incidence, predisposing factors, and prognosis. Rev Infect Dis **7:**133-142.
62. **Burton, D. R., and J. M. Woof.** 1992. Human antibody effector function. Adv Immunol **51:**1-84.
63. **Burton, R. L., and M. H. Nahm.** 2006. Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. Clin Vaccine Immunol **13:**1004-1009.
64. **Camara, M., G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1994. A neuraminidase from Streptococcus pneumoniae has the features of a surface protein. Infect Immun **62:**3688-3695.
65. **Camilli, R., A. Pantosti, and L. Baldassarri.** 2011. Contribution of serotype and genetic background to biofilm formation by Streptococcus pneumoniae. Eur J Clin Microbiol Infect Dis **30:**97-102.
66. **Carroll, M. C.** 2004. The complement system in regulation of adaptive immunity. Nat Immunol **5:**981-986.
67. **Casal, J., and D. Tarrago.** 2003. Immunity to Streptococcus pneumoniae: Factors affecting production and efficacy. Curr Opin Infect Dis **16:**219-224.
68. **Chiavolini, D., G. Memmi, T. Maggi, F. Iannelli, G. Pozzi, and M. R. Oggioni.** 2003. The three extra-cellular zinc metalloproteinases of Streptococcus pneumoniae have a different impact on virulence in mice. BMC Microbiol **3:**14.
69. **Childers, N. K., M. G. Bruce, and J. R. McGhee.** 1989. Molecular mechanisms of immunoglobulin A defense. Annu Rev Microbiol **43:**503-536.
70. **Claus, D. R., A. P. Osmand, and H. Gewurz.** 1976. Radioimmunoassay of human C-reactive protein and levels in normal sera. J Lab Clin Med **87:**120-128.
71. **Coffey, T. J., M. C. Enright, M. Daniels, J. K. Morona, R. Morona, W. Hryniewicz, J. C. Paton, and B. G. Spratt.** 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of Streptococcus pneumoniae. Mol Microbiol **27:**73-83.
72. **Cole, F. S., W. J. Matthews, Jr., T. H. Rossing, D. J. Gash, N. A. Lichtenberg, and J. E. Pennington.** 1983. Complement biosynthesis by human bronchoalveolar macrophages. Clin Immunol Immunopathol **27:**153-159.
73. **Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo.** 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A **75:**2458-2462.
74. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu Rev Microbiol **49:**711-745.
75. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-1322.
76. **Crain, M. J., W. D. Waltman, 2nd, J. S. Turner, J. Yother, D. F. Talkington, L. S. McDaniel, B. M. Gray, and D. E. Briles.** 1990. Pneumococcal surface

protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. Infect Immun **58:**3293-3299.

77. **Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen.** 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature **377:**435-438.
78. **Cundell, D. R., J. N. Weiser, J. Shen, A. Young, and E. I. Tuomanen.** 1995. Relationship between colonial morphology and adherence of Streptococcus pneumoniae. Infect Immun **63:**757-761.
79. **Cutts, F. T., S. M. Zaman, G. Enwere, S. Jaffar, O. S. Levine, J. B. Okoko, C. Oluwalana, A. Vaughan, S. K. Obaro, A. Leach, K. P. McAdam, E. Biney, M. Saaka, U. Onwuchekwa, F. Yallop, N. F. Pierce, B. M. Greenwood, and R. A. Adegbola.** 2005. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet **365:**1139-1146.
80. **Dagan, R., N. Givon-Lavi, O. Zamir, M. Sikuler-Cohen, L. Guy, J. Janco, P. Yagupsky, and D. Fraser.** 2002. Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis **185:**927-936.
81. **Dagan, R., H. Kayhty, T. Wuorimaa, M. Yaich, F. Bailleux, O. Zamir, and J. Eskola.** 2004. Tolerability and immunogenicity of an eleven valent mixed carrier Streptococcus pneumoniae capsular polysaccharide-diphtheria toxoid or tetanus protein conjugate vaccine in Finnish and Israeli infants. Pediatr Infect Dis J **23:**91-98.
82. **Dagan, R., R. Melamed, M. Muallem, L. Piglansky, D. Greenberg, O. Abramson, P. M. Mendelman, N. Bohidar, and P. Yagupsky.** 1996. Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. J Infect Dis **174:**1271-1278.
83. **Dahlback, B., and B. Hildebrand.** 1983. Degradation of human complement component C4b in the presence of the C4b-binding protein-protein S complex. Biochem J **209:**857-863.
84. **Dave, S., A. Brooks-Walter, M. K. Pangburn, and L. S. McDaniel.** 2001. PspC, a pneumococcal surface protein, binds human factor H. Infect Immun **69:**3435-3437.
85. **Dave, S., M. K. Pangburn, C. Pruitt, and L. S. McDaniel.** 2004. Interaction of human factor H with PspC of Streptococcus pneumoniae. Indian J Med Res **119 Suppl:**66-73.
86. **De, B. K., J. S. Sampson, E. W. Ades, R. C. Huebner, D. L. Jue, S. E. Johnson, M. Espina, A. R. Stinson, D. E. Briles, and G. M. Carlone.** 2000. Purification and characterization of Streptococcus pneumoniae palmitoylated pneumococcal surface adhesin A expressed in Escherichia coli. Vaccine **18:**1811-1821.

87. **De Lencastre, H., and A. Tomasz.** 2002. From ecological reservoir to disease: the nasopharynx, day-care centres and drug-resistant clones of Streptococcus pneumoniae. J Antimicrob Chemother **50 Suppl S2:**75-81.
88. **Dempsey, P. W., M. E. Allison, S. Akkaraju, C. C. Goodnow, and D. T. Fearon.** 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science **271:**348-350.
89. **Dieudonne-Vatran, A., S. Krentz, A. M. Blom, S. Meri, B. Henriques-Normark, K. Riesbeck, and B. Albiger.** 2009. Clinical isolates of Streptococcus pneumoniae bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. J Immunol **182:**7865-7877.
90. **Dintilhac, A., G. Alloing, C. Granadel, and J. P. Claverys.** 1997. Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol Microbiol **25:**727-739.
91. **DiScipio, R. G.** 1992. Formation and structure of the C5b-7 complex of the lytic pathway of complement. J Biol Chem **267:**17087-17094.
92. **Dodds, A. W., X. D. Ren, A. C. Willis, and S. K. Law.** 1996. The reaction mechanism of the internal thioester in the human complement component C4. Nature **379:**177-179.
93. **Drickamer, K., and M. E. Taylor.** 1993. Biology of animal lectins. Annu Rev Cell Biol **9:**237-264.
94. **Dworkin, M. S., J. W. Ward, D. L. Hanson, J. L. Jones, and J. E. Kaplan.** 2001. Pneumococcal disease among human immunodeficiency virus-infected persons: incidence, risk factors, and impact of vaccination. Clin Infect Dis **32:**794-800.
95. **Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli, and R. Landmann.** 2002. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis **186:**798-806.
96. **Ekdahl, K., I. Ahlinder, H. B. Hansson, E. Melander, S. Molstad, M. Soderstrom, and K. Persson.** 1997. Duration of nasopharyngeal carriage of penicillin-resistant Streptococcus pneumoniae: experiences from the South Swedish Pneumococcal Intervention Project. Clin Infect Dis **25:**1113-1117.
97. **Ekstrom, N., H. Ahman, J. Verho, J. Jokinen, M. Vakevainen, T. Kilpi, and H. Kayhty.** 2005. Kinetics and avidity of antibodies evoked by heptavalent pneumococcal conjugate vaccines PncCRM and PncOMPC in the Finnish Otitis Media Vaccine Trial. Infect Immun **73:**369-377.
98. **Ekstrom, N., M. Vakevainen, J. Verho, T. Kilpi, and H. Kayhty.** 2007. Functional antibodies elicited by two heptavalent pneumococcal conjugate vaccines in the Finnish Otitis Media Vaccine Trial. Infect Immun **75:**1794-1800.

99. **Elm, C., R. Braathen, S. Bergmann, R. Frank, J. P. Vaerman, C. S. Kaetzel, G. S. Chhatwal, F. E. Johansen, and S. Hammerschmidt.** 2004. Ectodomains 3 and 4 of human polymeric Immunoglobulin receptor (hpIgR) mediate invasion of Streptococcus pneumoniae into the epithelium. J Biol Chem **279:**6296-6304.
100. **Elm, C., M. Rohde, J. P. Vaerman, G. S. Chhatwal, and S. Hammerschmidt.** 2004. Characterization of the interaction of the pneumococcal surface protein SpsA with the human polymeric immunoglobulin receptor (hpIgR). Indian J Med Res **119 Suppl:**61-65.
101. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for Streptococcus pneumoniae: identification of clones associated with serious invasive disease. Microbiology **144 (Pt 11):**3049-3060.
102. **Eraklis, A. J., S. V. Kevy, L. K. Diamond, and R. E. Gross.** 1967. Hazard of overwhelming infection after splenectomy in childhood. New England Journal of Medicine **276:**1227-1229.
103. **Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Kayhty, P. Karma, R. Kohberger, G. Siber, and P. H. Makela.** 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med **344:**403-409.
104. **Esposito, S., S. Tansey, A. Thompson, A. Razmpour, J. Liang, T. R. Jones, G. Ferrera, A. Maida, G. Bona, C. Sabatini, L. Pugni, E. A. Emini, W. C. Gruber, D. A. Scott, and N. Principi.** 2010. Safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine compared to those of a 7-valent pneumococcal conjugate vaccine given as a three-dose series with routine vaccines in healthy infants and toddlers. Clin Vaccine Immunol **17:**1017-1026.
105. **Fearon, D. T., and W. W. Wong.** 1983. Complement ligand-receptor interactions that mediate biological responses. Annu Rev Immunol **1:**243-271.
106. **Feldman, C.** 2001. Pneumonia in the elderly. Med Clin North Am **85:**1441-1459.
107. **Feldman, C., T. J. Mitchell, P. W. Andrew, G. J. Boulnois, R. C. Read, H. C. Todd, P. J. Cole, and R. Wilson.** 1990. The effect of Streptococcus pneumoniae pneumolysin on human respiratory epithelium in vitro. Microb Pathog **9:**275-284.
108. **Figueroa, J. E., and P. Densen.** 1991. Infectious diseases associated with complement deficiencies. Clin Microbiol Rev **4:**359-395.
109. **Forneris, F., D. Ricklin, J. Wu, A. Tzekou, R. S. Wallace, J. D. Lambris, and P. Gros.** 2011. Structures of C3b in complex with factors B and D give insight into complement convertase formation. Science **330:**1816-1820.
110. **French, N.** 2003. Use of pneumococcal polysaccharide vaccines: no simple answers. J Infect **46:**78-86.
111. **Fujita, T.** 2002. Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol **2:**346-353.

112. **Garcia-Bustos, J. F., and A. Tomasz.** 1987. Teichoic acid-containing muropeptides from Streptococcus pneumoniae as substrates for the pneumococcal autolysin. J Bacteriol **169:**447-453.
113. **Garcia-Rodriguez, J. A., and M. J. Fresnadillo Martinez.** 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. J Antimicrob Chemother **50 Suppl S2:**59-73.
114. **Ghaffar, F., I. R. Friedland, and G. H. McCracken, Jr.** 1999. Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae. Pediatr Infect Dis J **18:**638-646.
115. **Gianfaldoni, C., S. Censini, M. Hilleringmann, M. Moschioni, C. Facciotti, W. Pansegrau, V. Masignani, A. Covacci, R. Rappuoli, M. A. Barocchi, and P. Ruggiero.** 2007. Streptococcus pneumoniae pilus subunits protect mice against lethal challenge. Infect Immun **75:**1059-1062.
116. **Giebink, G. S.** 2001. The prevention of pneumococcal disease in children. N Engl J Med **345:**1177-1183.
117. **Gilks, C. F., S. A. Ojoo, and R. J. Brindle.** 1996. Invasive pneumococcal disease in a cohort of predominantly HIV-1 infected female sex-workers in Nairobi, Kenya. Lancet **347:**718-723.
118. **Gill, C. J., V. Mwanakasale, M. P. Fox, R. Chilengi, M. Tembo, M. Nsofwa, V. Chalwe, L. Mwananyanda, D. Mukwamataba, B. Malilwe, D. Champo, W. B. Macleod, D. M. Thea, and D. H. Hamer.** 2008. Impact of human immunodeficiency virus infection on Streptococcus pneumoniae colonization and seroepidemiology among Zambian women. J Infect Dis **197:**1000-1005.
119. **Godfroid, F., P. Hermand, V. Verlant, P. Denoel, and J. T. Poolman.** 2010. Preclinical evaluation of the Pht proteins as potential cross-protective pneumococcal vaccine antigens. Infect Immun.
120. **Goetghebuer, T., T. E. West, V. Wermenbol, A. L. Cadbury, P. Milligan, N. Lloyd-Evans, R. A. Adegbola, E. K. Mulholland, B. M. Greenwood, and M. W. Weber.** 2000. Outcome of meningitis caused by Streptococcus pneumoniae and Haemophilus influenzae type b in children in The Gambia. Trop Med Int Health **5:**207-213.
121. **Goldblatt, D., M. Hussain, N. Andrews, L. Ashton, C. Virta, A. Melegaro, R. Pebody, R. George, A. Soininen, J. Edmunds, N. Gay, H. Kayhty, and E. Miller.** 2005. Antibody responses to nasopharyngeal carriage of Streptococcus pneumoniae in adults: a longitudinal household study. J Infect Dis **192:**387-393.
122. **Gordon, D. L., G. M. Johnson, and M. K. Hostetter.** 1986. Ligand-receptor interactions in the phagocytosis of virulent Streptococcus pneumoniae by polymorphonuclear leukocytes. J Infect Dis **154:**619-626.
123. **Gordon, D. L., J. Rice, J. J. Finlay-Jones, P. J. McDonald, and M. K. Hostetter.** 1988. Analysis of C3 deposition and degradation on bacterial surfaces after opsonization. J Infect Dis **157:**697-704.

124. **Gordon, S. B., G. R. Irving, R. A. Lawson, M. E. Lee, and R. C. Read.** 2000. Intracellular trafficking and killing of Streptococcus pneumoniae by human alveolar macrophages are influenced by opsonins. Infect Immun **68:**2286-2293.
125. **Gordon, S. B., and R. C. Read.** 2002. Macrophage defences against respiratory tract infections. Br Med Bull **61:**45-61.
126. **Granat, S. M., Z. Mia, J. Ollgren, E. Herva, M. Das, L. Piirainen, K. Auranen, and P. H. Makela.** 2007. Longitudinal study on pneumococcal carriage during the first year of life in Bangladesh. Pediatr Infect Dis J **26:**319-324.
127. **Gray, B. M., G. M. Converse, 3rd, and H. C. Dillon, Jr.** 1980. Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis **142:**923-933.
128. **Gray, B. M., and H. C. Dillon, Jr.** 1989. Natural history of pneumococcal infections. Pediatr Infect Dis J **8:**S23-25.
129. **Gwaltney, J. M., Jr., W. M. Scheld, M. A. Sande, and A. Sydnor.** 1992. The microbial etiology and antimicrobial therapy of adults with acute community-acquired sinusitis: a fifteen-year experience at the University of Virginia and review of other selected studies. J Allergy Clin Immunol **90:**457-461; discussion 462.
130. **Hakansson, A., H. Roche, S. Mirza, L. S. McDaniel, A. Brooks-Walter, and D. E. Briles.** 2001. Characterization of binding of human lactoferrin to pneumococcal surface protein A. Infect Immun **69:**3372-3381.
131. **Hamel, J., N. Charland, I. Pineau, C. Ouellet, S. Rioux, D. Martin, and B. R. Brodeur.** 2004. Prevention of pneumococcal disease in mice immunized with conserved surface-accessible proteins. Infect Immun **72:**2659-2670.
132. **Hammerschmidt, S., V. Agarwal, A. Kunert, S. Haelbich, C. Skerka, and P. F. Zipfel.** 2007. The host immune regulator factor H interacts via two contact sites with the PspC protein of Streptococcus pneumoniae and mediates adhesion to host epithelial cells. J Immunol **178:**5848-5858.
133. **Hammerschmidt, S., G. Bethe, P. H. Remane, and G. S. Chhatwal.** 1999. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of Streptococcus pneumoniae. Infect Immun **67:**1683-1687.
134. **Hammerschmidt, S., S. R. Talay, P. Brandtzaeg, and G. S. Chhatwal.** 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. Mol Microbiol **25:**1113-1124.
135. **Hammerschmidt, S., S. Wolff, A. Hocke, S. Rosseau, E. Muller, and M. Rohde.** 2005. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect Immun **73:**4653-4667.
136. **Hanage, W. P., K. Auranen, R. Syrjanen, E. Herva, P. H. Makela, T. Kilpi, and B. G. Spratt.** 2004. Ability of pneumococcal serotypes and clones to cause acute otitis media: implications for the prevention of otitis media by conjugate vaccines. Infect Immun **72:**76-81.

137. **Hanage, W. P., T. H. Kaijalainen, R. K. Syrjanen, K. Auranen, M. Leinonen, P. H. Makela, and B. G. Spratt.** 2005. Invasiveness of serotypes and clones of Streptococcus pneumoniae among children in Finland. Infect Immun **73:**431-435.
138. **Harboe, Z. B., R. W. Thomsen, A. Riis, P. Valentiner-Branth, J. J. Christensen, L. Lambertsen, K. A. Krogfelt, H. B. Konradsen, and T. L. Benfield.** 2009. Pneumococcal serotypes and mortality following invasive pneumococcal disease: a population-based cohort study. PLoS Med **6:**e1000081.
139. **Harding, C. V., R. W. Roof, P. M. Allen, and E. R. Unanue.** 1991. Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex. Proc Natl Acad Sci USA **88**.
140. **Harlyk, C., J. McCourt, G. Bordin, A. R. Rodriguez, and A. van der Eeckhout.** 1997. Determination of copper, zinc and iron in broncho-alveolar lavages by atomic absorption spectroscopy. J Trace Elem Med Biol **11:**137-142.
141. **Hausdorff, W. P., J. Bryant, P. R. Paradiso, and G. R. Siber.** 2000. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis **30:**100-121.
142. **Hausdorff, W. P., G. Yothers, R. Dagan, T. Kilpi, S. I. Pelton, R. Cohen, M. R. Jacobs, S. L. Kaplan, C. Levy, E. L. Lopez, E. O. Mason, Jr., V. Syriopoulou, B. Wynne, and J. Bryant.** 2002. Multinational study of pneumococcal serotypes causing acute otitis media in children. Pediatr Infect Dis J **21:**1008-1016.
143. **Hava, D. L., and A. Camilli.** 2002. Large-scale identification of serotype 4 Streptococcus pneumoniae virulence factors. Mol Microbiol **45:**1389-1406.
144. **Hava, D. L., J. LeMieux, and A. Camilli.** 2003. From nose to lung: the regulation behind Streptococcus pneumoniae virulence factors. Mol Microbiol **50:**1103-1110.
145. **He, X., and L. S. McDaniel.** 2007. The genetic background of Streptococcus pneumoniae affects protection in mice immunized with PspA. FEMS Microbiol Lett **269:**189-195.
146. **Helmy, K. Y., K. J. Katschke, Jr., N. N. Gorgani, N. M. Kljavin, J. M. Elliott, L. Diehl, S. J. Scales, N. Ghilardi, and M. van Lookeren Campagne.** 2006. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. Cell **124:**915-927.
147. **Hennessy, T. W., R. J. Singleton, L. R. Bulkow, D. L. Bruden, D. A. Hurlburt, D. Parks, M. Moore, A. J. Parkinson, A. Schuchat, and J. C. Butler.** 2005. Impact of heptavalent pneumococcal conjugate vaccine on invasive disease, antimicrobial resistance and colonization in Alaska Natives: progress towards elimination of a health disparity. Vaccine **23:**5464-5473.
148. **Hicks, L. A., L. H. Harrison, B. Flannery, J. L. Hadler, W. Schaffner, A. S. Craig, D. Jackson, A. Thomas, B. Beall, R. Lynfield, A. Reingold, M. M. Farley, and C. G. Whitney.** 2007. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004. J Infect Dis **196:**1346-1354.

149. **Hill, P. C., Y. B. Cheung, A. Akisanya, K. Sankareh, G. Lahai, B. M. Greenwood, and R. A. Adegbola.** 2008. Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian infants: a longitudinal study. Clin Infect Dis **46:**807-814.
150. **Hirst, R. A., A. Kadioglu, C. O'Callaghan, and P. W. Andrew.** 2004. The role of pneumolysin in pneumococcal pneumonia and meningitis. Clin Exp Immunol **138:**195-201.
151. **Hogberg, L., P. Geli, H. Ringberg, E. Melander, M. Lipsitch, and K. Ekdahl.** 2007. Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. J Clin Microbiol **45:**948-952.
152. **Hollingshead, S. K., R. Becker, and D. E. Briles.** 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun **68:**5889-5900.
153. **Honkanen, P. O., T. Keistinen, L. Miettinen, E. Herva, U. Sankilampi, E. Laara, M. Leinonen, S. L. Kivela, and P. H. Makela.** 1999. Incremental effectiveness of pneumococcal vaccine on simultaneously administered influenza vaccine in preventing pneumonia and pneumococcal pneumonia among persons aged 65 years or older. Vaccine **17:**2493-2500.
154. **Hosea, S. W., E. J. Brown, and M. M. Frank.** 1980. The critical role of complement in experimental pneumococcal sepsis. J Infect Dis **142:**903-909.
155. **Hostetter, M. K.** 1999. Opsonic and nonopsonic interactions of C3 with Streptococcus pneumoniae. Microb Drug Resist **5:**85-89.
156. **Hostetter, M. K.** 1986. Serotypic variations among virulent pneumococci in deposition and degradation of covalently bound C3b: implications for phagocytosis and antibody production. J Infect Dis **153:**682-693.
157. **Hostetter, M. K., R. A. Krueger, and D. J. Schmeling.** 1984. The biochemistry of opsonization: central role of the reactive thiolester of the third component of complement. J Infect Dis **150:**653-661.
158. **Hostetter, M. K., M. L. Thomas, F. S. Rosen, and B. F. Tack.** 1982. Binding of C3b proceeds by a transesterification reaction at the thiolester site. Nature **298:**72-75.
159. **Houldsworth, S., P. W. Andrew, and T. J. Mitchell.** 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. Infect Immun **62:**1501-1503.
160. **Hourcade, D., M. K. Liszewski, M. Krych-Goldberg, and J. P. Atkinson.** 2000. Functional domains, structural variations and pathogen interactions of MCP, DAF and CR1. Immunopharmacology **49:**103-116.
161. **Huang, S. S., R. Platt, S. L. Rifas-Shiman, S. I. Pelton, D. Goldmann, and J. A. Finkelstein.** 2005. Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. Pediatrics **116:**e408-413.

162. **Hyams, C., E. Camberlein, J. M. Cohen, K. Bax, and J. S. Brown.** 2010. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun **78:**704-715.
163. **Hyams, C., J. Yuste, K. Bax, E. Camberlein, J. N. Weiser, and J. S. Brown.** 2010. Streptococcus pneumoniae resistance to complement-mediated immunity is dependent on the capsular serotype. Infect Immun **78:**716-725.
164. **Iannelli, F., M. R. Oggioni, and G. Pozzi.** 2002. Allelic variation in the highly polymorphic locus pspC of Streptococcus pneumoniae. Gene **284:**63-71.
165. **Jackson, L. A., K. M. Neuzil, O. Yu, P. Benson, W. E. Barlow, A. L. Adams, C. A. Hanson, L. D. Mahoney, D. K. Shay, and W. W. Thompson.** 2003. Effectiveness of pneumococcal polysaccharide vaccine in older adults. N Engl J Med **348:**1747-1755.
166. **Jacobs, M. R., D. Felmingham, P. C. Appelbaum, and R. N. Gruneberg.** 2003. The Alexander Project 1998-2000: susceptibility of pathogens isolated from community-acquired respiratory tract infection to commonly used antimicrobial agents. J Antimicrob Chemother **52:**229-246.
167. **Jado, I., A. Fenoll, J. Casal, and A. Perez.** 2001. Identification of the psaA gene, coding for pneumococcal surface adhesin A, in viridans group streptococci other than Streptococcus pneumoniae. Clin Diagn Lab Immunol **8:**895-898.
168. **Janoff, E. N., and J. B. Rubins.** 1997. Invasive pneumococcal disease in the immunocompromised host. Microb Drug Resist **3:**215-232.
169. **Janulczyk, R., F. Iannelli, A. G. Sjoholm, G. Pozzi, and L. Bjorck.** 2000. Hic, a novel surface protein of Streptococcus pneumoniae that interferes with complement function. J Biol Chem **275:**37257-37263.
170. **Jarva, H., R. Janulczyk, J. Hellwage, P. F. Zipfel, L. Bjorck, and S. Meri.** 2002. Streptococcus pneumoniae evades complement attack and opsonophagocytosis by expressing the pspC locus-encoded Hic protein that binds to short consensus repeats 8-11 of factor H. J Immunol **168:**1886-1894.
171. **Jarva, H., T. S. Jokiranta, J. Hellwage, P. F. Zipfel, and S. Meri.** 1999. Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8-11. J Immunol **163:**3957-3962.
172. **Jarva, H., T. S. Jokiranta, R. Wurzner, and S. Meri.** 2003. Complement resistance mechanisms of streptococci. Mol Immunol **40:**95-107.
173. **Jefferies, J. M., A. S. Tocheva, H. Rubery, J. Bennett, J. Garland, M. Christodoulides, S. N. Faust, A. Smith, T. J. Mitchell, and S. C. Clarke.** 2010. Identification of novel pneumolysin alleles from paediatric carriage isolates of Streptococcus pneumoniae. J Med Microbiol **59:**808-814.
174. **Jodar, L., J. Butler, G. Carlone, R. Dagan, D. Goldblatt, H. Kayhty, K. Klugman, B. Plikaytis, G. Siber, R. Kohberger, I. Chang, and T. Cherian.**

2003. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. Vaccine **21:**3265-3272.

175. **Johnson, S. E., L. Rubin, S. Romero-Steiner, J. K. Dykes, L. B. Pais, A. Rizvi, E. Ades, and G. M. Carlone.** 1999. Correlation of opsonophagocytosis and passive protection assays using human anticapsular antibodies in an infant mouse model of bacteremia for Streptococcus pneumoniae. J Infect Dis **180:**133-140.
176. **Jokinen, J. T., H. Ahman, T. M. Kilpi, P. H. Makela, and M. H. Kayhty.** 2004. Concentration of antipneumococcal antibodies as a serological correlate of protection: an application to acute otitis media. J Infect Dis **190:**545-550.
177. **Jokiranta, T. S., P. F. Zipfel, J. Hakulinen, S. Kuhn, M. K. Pangburn, J. D. Tamerius, and S. Meri.** 1996. Analysis of the recognition mechanism of the alternative pathway of complement by monoclonal anti-factor H antibodies: evidence for multiple interactions between H and surface bound C3b. FEBS Lett **393:**297-302.
178. **Kadioglu, A., S. Taylor, F. Iannelli, G. Pozzi, T. J. Mitchell, and P. W. Andrew.** 2002. Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type. Infect Immun **70:**2886-2890.
179. **Kadioglu, A., J. N. Weiser, J. C. Paton, and P. W. Andrew.** 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol **6:**288-301.
180. **Kang, Y. S., Y. Do, H. K. Lee, S. H. Park, C. Cheong, R. M. Lynch, J. M. Loeffler, R. M. Steinman, and C. G. Park.** 2006. A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. Cell **125:**47-58.
181. **Kanneganti, T. D., M. Lamkanfi, and G. Nunez.** 2007. Intracellular NOD-like receptors in host defense and disease. Immunity **27:**549-559.
182. **Kelly, T., J. P. Dillard, and J. Yother.** 1994. Effect of genetic switching of capsular type on virulence of Streptococcus pneumoniae. Infect Immun **62:**1813-1819.
183. **Kerr, A. R., G. K. Paterson, J. McCluskey, F. Iannelli, M. R. Oggioni, G. Pozzi, and T. J. Mitchell.** 2006. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. Infect Immun **74:**5319-5324.
184. **Kerr, A. R., G. K. Paterson, A. Riboldi-Tunnicliffe, and T. J. Mitchell.** 2005. Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. Infect Immun **73:**4245-4252.
185. **Kieninger, D. M., K. Kueper, K. Steul, C. Juergens, N. Ahlers, S. Baker, K. U. Jansen, C. Devlin, W. C. Gruber, E. A. Emini, and D. A. Scott.** 2010. Safety, tolerability, and immunologic noninferiority of a 13-valent pneumococcal

conjugate vaccine compared to a 7-valent pneumococcal conjugate vaccine given with routine pediatric vaccinations in Germany. Vaccine **28:**4192-4203.

186. **Kilian, M., J. Reinholdt, H. Lomholt, K. Poulsen, and E. V. Frandsen.** 1996. Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. Apmis **104:**321-338.
187. **Kilpi, T., H. Ahman, J. Jokinen, K. S. Lankinen, A. Palmu, H. Savolainen, M. Gronholm, M. Leinonen, T. Hovi, J. Eskola, H. Kayhty, N. Bohidar, J. C. Sadoff, and P. H. Makela.** 2003. Protective Efficacy of a Second Pneumococcal Conjugate Vaccine against Pneumococcal Acute Otitis Media in Infants and Children: Randomized, Controlled Trial of a 7-Valent Pneumococcal Polysaccharide-Meningococcal Outer Membrane Protein Complex Conjugate Vaccine in 1666 Children. Clin Infect Dis **37:**1155-1164.
188. **Kilpi, T., E. Herva, T. Kaijalainen, R. Syrjanen, and A. K. Takala.** 2001. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. Pediatr Infect Dis J **20:**654-662.
189. **Kim, J. O., S. Romero-Steiner, U. B. Sorensen, J. Blom, M. Carvalho, S. Barnard, G. Carlone, and J. N. Weiser.** 1999. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of Streptococcus pneumoniae. Infect Immun **67:**2327-2333.
190. **Kim, J. O., and J. N. Weiser.** 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae. J Infect Dis **177:**368-377.
191. **Kim, J. S., E. R. Laskowich, R. G. Arumugham, R. E. Kaiser, and G. J. MacMichael.** 2005. Determination of saccharide content in pneumococcal polysaccharides and conjugate vaccines by GC-MSD. Anal Biochem **347:**262-274.
192. **Klickstein, L. B., S. F. Barbashov, T. Liu, R. M. Jack, and A. Nicholson-Weller.** 1997. Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity **7:**345-355.
193. **Klugman, K. P., S. A. Madhi, and C. Feldman.** 2007. HIV and pneumococcal disease. Curr Opin Infect Dis **20:**11-15.
194. **Klugman, K. P., S. A. Madhi, R. E. Huebner, R. Kohberger, N. Mbelle, and N. Pierce.** 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med **349:**1341-1348.
195. **Knapp, S., C. W. Wieland, C. van 't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll.** 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. J Immunol **172:**3132-3138.
196. **Knight-Madden, J., and G. R. Serjeant.** 2001. Invasive pneumococcal disease in homozygous sickle-cell disease: Jamaican experience 1973-1997. Journal of Pediatrics **138:**65-70.

197. **Krych-Goldberg, M., and J. P. Atkinson.** 2001. Structure-function relationships of complement receptor type 1. Immunol Rev **180:**112-122.
198. **Kumar, H., T. Kawai, and S. Akira.** 2009. Pathogen recognition in the innate immune response. Biochem J **420:**1-16.
199. **Lachmann, P. J., and N. C. Hughes-Jones.** 1984. Initiation of complement activation. Springer Semin Immunopathol **7:**143-162.
200. **Lagos, R., A. Munoz, O. San Martin, A. Maldonado, J. C. Hormazabal, W. C. Blackwelder, and M. M. Levine.** 2008. Age- and serotype-specific pediatric invasive pneumococcal disease: insights from systematic surveillance in Santiago, Chile, 1994--2007. J Infect Dis **198:**1809-1817.
201. **Lambris, J. D., D. Ricklin, and B. V. Geisbrecht.** 2008. Complement evasion by human pathogens. Nat Rev Microbiol **6:**132-142.
202. **Lanie, J. A., W. L. Ng, K. M. Kazmierczak, T. M. Andrzejewski, T. M. Davidsen, K. J. Wayne, H. Tettelin, J. I. Glass, and M. E. Winkler.** 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of Streptococcus pneumoniae and comparison with that of unencapsulated laboratory strain R6. J Bacteriol **189:**38-51.
203. **Law, S. K., N. A. Lichtenberg, and R. P. Levine.** 1980. Covalent binding and hemolytic activity of complement proteins. Proc Natl Acad Sci U S A **77:**7194-7198.
204. **Law, S. K., T. M. Minich, and R. P. Levine.** 1981. Binding reaction between the third human complement protein and small molecules. Biochemistry **20:**7457-7463.
205. **Lawrence, M. C., P. A. Pilling, V. C. Epa, A. M. Berry, A. D. Ogunniyi, and J. C. Paton.** 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure **6:**1553-1561.
206. **Leach, A. J., J. B. Boswell, V. Asche, T. G. Nienhuys, and J. D. Mathews.** 1994. Bacterial colonization of the nasopharynx predicts very early onset and persistence of otitis media in Australian aboriginal infants. Pediatr Infect Dis J **13:**983-989.
207. **Lee, C. J., S. D. Banks, and J. P. Li.** 1991. Virulence, immunity, and vaccine related to Streptococcus pneumoniae. Crit Rev Microbiol **18:**89-114.
208. **Leino, T., F. Hoti, R. Syrjanen, A. Tanskanen, and K. Auranen.** 2008. Clustering of serotypes in a longitudinal study of Streptococcus pneumoniae carriage in three day care centres. BMC Infect Dis **8:**173.
209. **LeMieux, J., D. L. Hava, A. Basset, and A. Camilli.** 2006. RrgA and RrgB are components of a multisubunit pilus encoded by the Streptococcus pneumoniae rlrA pathogenicity islet. Infect Immun **74:**2453-2456.
210. **Lesavre, P. H., T. E. Hugli, A. F. Esser, and H. J. Muller-Eberhard.** 1979. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. J Immunol **123:**529-534.

211. **Lesavre, P. H., and H. J. Muller-Eberhard.** 1978. Mechanism of action of factor D of the alternative complement pathway. J Exp Med **148:**1498-1509.
212. **Lexau, C. A., R. Lynfield, R. Danila, T. Pilishvili, R. Facklam, M. M. Farley, L. H. Harrison, W. Schaffner, A. Reingold, N. M. Bennett, J. Hadler, P. R. Cieslak, and C. G. Whitney.** 2005. Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. JAMA **294:**2043-2051.
213. **Li, J., D. T. Glover, A. J. Szalai, S. K. Hollingshead, and D. E. Briles.** 2007. PspA and PspC minimize immune adherence and transfer of pneumococci from erythrocytes to macrophages through their effects on complement activation. Infect Immun **75:**5877-5885.
214. **Linares, J., C. Ardanuy, R. Pallares, and A. Fenoll.** 2010. Changes in antimicrobial resistance, serotypes and genotypes in Streptococcus pneumoniae over a 30-year period. Clin Microbiol Infect **16:**402-410.
215. **Linder, A., S. Hollingshead, R. Janulczyk, B. Christensson, and P. Akesson.** 2007. Human antibody response towards the pneumococcal surface proteins PspA and PspC during invasive pneumococcal infection. Vaccine **25:**341-345.
216. **Lipsitch, M., K. O'Neill, D. Cordy, B. Bugalter, K. Trzcinski, C. M. Thompson, R. Goldstein, S. Pelton, H. Huot, V. Bouchet, R. Reid, M. Santosham, and K. L. O'Brien.** 2007. Strain characteristics of Streptococcus pneumoniae carriage and invasive disease isolates during a cluster-randomized clinical trial of the 7-valent pneumococcal conjugate vaccine. J Infect Dis **196:**1221-1227.
217. **Lipsitch, M., C. G. Whitney, E. Zell, T. Kaijalainen, R. Dagan, and R. Malley.** 2005. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med **2:**e15.
218. **Lomholt, H.** 1995. Evidence of recombination and an antigenically diverse immunoglobulin A1 protease among strains of Streptococcus pneumoniae. Infect Immun **63:**4238-4243.
219. **Loos, M., and F. Clas.** 1987. Antibody-independent killing of gram-negative bacteria via the classical pathway of complement. Immunol Lett **14:**203-208.
220. **Lu, L., Y. Ma, and J. R. Zhang.** 2006. Streptococcus pneumoniae recruits complement factor H through the amino terminus of CbpA. J Biol Chem **281:**15464-15474.
221. **Lu, L., Z. Ma, T. S. Jokiranta, A. R. Whitney, F. R. DeLeo, and J. R. Zhang.** 2008. Species-specific interaction of Streptococcus pneumoniae with human complement factor H. J Immunol **181:**7138-7146.
222. **Lu, Y. J., J. Gross, D. Bogaert, A. Finn, L. Bagrade, Q. Zhang, J. K. Kolls, A. Srivastava, A. Lundgren, S. Forte, C. M. Thompson, K. F. Harney, P. W. Anderson, M. Lipsitch, and R. Malley.** 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathog **4:**e1000159.

223. **Lucero, M. G., H. Nohynek, G. Williams, V. Tallo, E. A. Simoes, S. Lupisan, D. Sanvictores, S. Forsyth, T. Puumalainen, J. Ugpo, M. Lechago, M. de Campo, E. Abucejo-Ladesma, L. Sombrero, A. Nissinen, A. Soininen, P. Ruutu, I. Riley, and H. P. Makela.** 2009. Efficacy of an 11-valent pneumococcal conjugate vaccine against radiologically confirmed pneumonia among children less than 2 years of age in the Philippines: a randomized, double-blind, placebo-controlled trial. Pediatr Infect Dis J **28:**455-462.
224. **Lysenko, E. S., R. S. Lijek, S. P. Brown, and J. N. Weiser.** 2010. Within-host competition drives selection for the capsule virulence determinant of Streptococcus pneumoniae. Curr Biol **20:**1222-1226.
225. **Lysenko, E. S., A. J. Ratner, A. L. Nelson, and J. N. Weiser.** 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog **1:**e1.
226. **Mac, L. C., and M. R. Kraus.** 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. J Exp Med **92:**1-9.
227. **Macleod, C. M., R. G. Hodges, M. Heidelberger, and W. G. Bernhard.** 1945. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. J Exp Med **82:**445-465.
228. **Magee, A. D., and J. Yother.** 2001. Requirement for capsule in colonization by Streptococcus pneumoniae. Infect Immun **69:**3755-3761.
229. **Malley, R.** 2010. Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. J Mol Med **88:**135-142.
230. **Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock.** 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A **100:**1966-1971.
231. **Malley, R., M. Lipsitch, A. Stack, R. Saladino, G. Fleisher, S. Pelton, C. Thompson, D. Briles, and P. Anderson.** 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun **69:**4870-4873.
232. **Malley, R., K. Trzcinski, A. Srivastava, C. M. Thompson, P. W. Anderson, and M. Lipsitch.** 2005. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc Natl Acad Sci U S A **102:**4848-4853.
233. **Marra, A., S. Lawson, J. S. Asundi, D. Brigham, and A. E. Hromockyj.** 2002. In vivo characterization of the psa genes from Streptococcus pneumoniae in multiple models of infection. Microbiology **148:**1483-1491.
234. **Marriott, H. M., T. J. Mitchell, and D. H. Dockrell.** 2008. Pneumolysin: a double-edged sword during the host-pathogen interaction. Curr Mol Med **8:**497-509.

235. **Matthias, K. A., A. M. Roche, A. J. Standish, M. Shchepetov, and J. N. Weiser.** 2008. Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of Streptococcus pneumoniae. J Immunol **180:**6246-6254.
236. **Mbelle, N., R. E. Huebner, A. D. Wasas, A. Kimura, I. Chang, and K. P. Klugman.** 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J Infect Dis **180:**1171-1176.
237. **McCool, T. L., T. R. Cate, G. Moy, and J. N. Weiser.** 2002. The immune response to pneumococcal proteins during experimental human carriage. J Exp Med **195:**359-365.
238. **McDaniel, L. S., J. Yother, M. Vijayakumar, L. McGarry, W. R. Guild, and D. E. Briles.** 1987. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). J Exp Med **165:**381-394.
239. **Melin, M. M., S. K. Hollingshead, D. E. Briles, M. I. Lahdenkari, T. M. Kilpi, and H. M. Käyhty.** 2008. Development of Antibodies to PspA Families 1 and 2 in Children after Exposure to *Streptococcus pneumoniae*. Clinical and Vaccine Immunology **15**.
240. **Meri, S.** 2007. Loss of self-control in the complement system and innate autoreactivity. Ann N Y Acad Sci **1109:**93-105.
241. **Mestecky, J.** 2005. Mucosal Immunology, 3rd edition ed, vol. Chapter 5. Elsevier Academic Press.
242. **Miyaji, E. N., D. M. Ferreira, A. P. Lopes, M. C. Brandileone, W. O. Dias, and L. C. Leite.** 2002. Analysis of serum cross-reactivity and cross-protection elicited by immunization with DNA vaccines against *Streptococcus pneumoniae* expressing PspA fragments from different clades. Infect Immun **70:**5086-5090.
243. **Mold, C., H. Gewurz, and T. W. Du Clos.** 1999. Regulation of complement activation by C-reactive protein. Immunopharmacology **42:**23-30.
244. **Mold, C., C. P. Rodgers, R. L. Kaplan, and H. Gewurz.** 1982. Binding of human C-reactive protein to bacteria. Infect Immun **38:**392-395.
245. **Molina, H., T. Kinoshita, C. B. Webster, and V. M. Holers.** 1994. Analysis of C3b/C3d binding sites and factor I cofactor regions within mouse complement receptors 1 and 2. J Immunol **153:**789-795.
246. **Montgomery, J. M., D. Lehmann, T. Smith, A. Michael, B. Joseph, T. Lupiwa, C. Coakley, V. Spooner, B. Best, I. D. Riley, and et al.** 1990. Bacterial colonization of the upper respiratory tract and its association with acute lower respiratory tract infections in Highland children of Papua New Guinea. Rev Infect Dis **12 Suppl 8:**S1006-1016.
247. **Moore, M. R., T. B. Hyde, T. W. Hennessy, D. J. Parks, A. L. Reasonover, M. Harker-Jones, J. Gove, D. L. Bruden, K. Rudolph, A. Parkinson, J. C. Butler, and A. Schuchat.** 2004. Impact of a Conjugate Vaccine on Community-Wide Carriage of Nonsusceptible Streptococcus pneumoniae in Alaska. J Infect Dis **190:**2031-2038.

248. **Morrison, K. E., D. Lake, J. Crook, G. M. Carlone, E. Ades, R. Facklam, and J. S. Sampson.** 2000. Confirmation of psaA in all 90 serotypes of Streptococcus pneumoniae by PCR and potential of this assay for identification and diagnosis. J Clin Microbiol **38:**434-437.
249. **Moschioni, M., C. Donati, A. Muzzi, V. Masignani, S. Censini, W. P. Hanage, C. J. Bishop, J. N. Reis, S. Normark, B. Henriques-Normark, A. Covacci, R. Rappuoli, and M. A. Barocchi.** 2008. Streptococcus pneumoniae contains 3 rlrA pilus variants that are clonally related. J Infect Dis **197:**888-896.
250. **Moscoso, M., E. Garcia, and R. Lopez.** 2006. Biofilm formation by Streptococcus pneumoniae: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J Bacteriol **188:**7785-7795.
251. **Mostov, K. E.** 1994. Transepithelial transport of immunoglobulins. Annu Rev Immunol **12:**63-84.
252. **Mulholland, K.** 1999. Magnitude of the problem of childhood pneumonia. Lancet **354:**590-592.
253. **Musher, D. M.** 2003. How contagious are common respiratory tract infections? N Engl J Med **348:**1256-1266.
254. **Musher, D. M.** 1992. Infections caused by Streptococcus pneumoniae: clinical spectrum, pathogenesis, immunity, and treatment. Clin Infect Dis **14:**801-807.
255. **Nauta, A. J., B. Bottazzi, A. Mantovani, G. Salvatori, U. Kishore, W. J. Schwaeble, A. R. Gingras, S. Tzima, F. Vivanco, J. Egido, O. Tijsma, E. C. Hack, M. R. Daha, and A. Roos.** 2003. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. Eur J Immunol **33:**465-473.
256. **Neeleman, C., S. P. Geelen, P. C. Aerts, M. R. Daha, T. E. Mollnes, J. J. Roord, G. Posthuma, H. van Dijk, and A. Fleer.** 1999. Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by the binding of complement regulatory protein factor H. Infect Immun **67:**4517-4524.
257. **Nelson, A. L., J. Ries, F. Bagnoli, S. Dahlberg, S. Falker, S. Rounioja, J. Tschop, E. Morfeldt, I. Ferlenghi, M. Hilleringmann, D. W. Holden, R. Rappuoli, S. Normark, M. A. Barocchi, and B. Henriques-Normark.** 2007. RrgA is a pilus-associated adhesin in Streptococcus pneumoniae. Mol Microbiol **66:**329-340.
258. **Nelson, A. L., A. M. Roche, J. M. Gould, K. Chim, A. J. Ratner, and J. N. Weiser.** 2007. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun **75:**83-90.
259. **Nesin, M., M. Ramirez, and A. Tomasz.** 1998. Capsular transformation of a multidrug-resistant Streptococcus pneumoniae in vivo. J Infect Dis **177:**707-713.
260. **Novak, R., J. S. Braun, E. Charpentier, and E. Tuomanen.** 1998. Penicillin tolerance genes of Streptococcus pneumoniae: the ABC-type manganese permease complex Psa. Mol Microbiol **29:**1285-1296.

261. **O'Brien, K. L., A. B. David, A. Chandran, L. H. Moulton, R. Reid, R. Weatherholtz, and M. Santosham.** 2008. Randomized, controlled trial efficacy of pneumococcal conjugate vaccine against otitis media among Navajo and White Mountain Apache infants. Pediatr Infect Dis J **27:**71-73.
262. **O'Brien, K. L., L. H. Moulton, R. Reid, R. Weatherholtz, J. Oski, L. Brown, G. Kumar, A. Parkinson, D. Hu, J. Hackell, I. Chang, R. Kohberger, G. Siber, and M. Santosham.** 2003. Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. Lancet **362:**355-361.
263. **Obaro, S., and R. Adegbola.** 2002. The pneumococcus: carriage, disease and conjugate vaccines. J Med Microbiol **51:**98-104.
264. **Obaro, S. K., R. A. Adegbola, W. A. Banya, and B. M. Greenwood.** 1996. Carriage of pneumococci after pneumococcal vaccination. Lancet **348:**271-272.
265. **Ochs, M. M., W. Bartlett, D. E. Briles, B. Hicks, A. Jurkuvenas, P. Lau, B. Ren, and A. Millar.** 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition on Streptococcus pneumoniae. Microb Pathog **44:**204-214.
266. **Oggioni, M. R., C. Trappetti, A. Kadioglu, M. Cassone, F. Iannelli, S. Ricci, P. W. Andrew, and G. Pozzi.** 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol **61:**1196-1210.
267. **Ogunniyi, A. D., R. L. Folland, D. E. Briles, S. K. Hollingshead, and J. C. Paton.** 2000. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infect Immun **68:**3028-3033.
268. **Ogunniyi, A. D., M. Grabowicz, D. E. Briles, J. Cook, and J. C. Paton.** 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of Streptococcus pneumoniae. Infect Immun **75:**350-357.
269. **Ogunniyi, A. D., M. Grabowicz, L. K. Mahdi, J. Cook, D. L. Gordon, T. A. Sadlon, and J. C. Paton.** 2009. Pneumococcal histidine triad proteins are regulated by the Zn2+-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. Faseb J **23:**731-738.
270. **Ogunniyi, A. D., K. S. LeMessurier, R. M. Graham, J. M. Watt, D. E. Briles, U. H. Stroeher, and J. C. Paton.** 2007. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. Infect Immun **75:**1843-1851.
271. **Orihuela, C. J., G. Gao, K. P. Francis, J. Yu, and E. I. Tuomanen.** 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J Infect Dis **190:**1661-1669.

272. **Orihuela, C. J., J. N. Radin, J. E. Sublett, G. Gao, D. Kaushal, and E. I. Tuomanen.** 2004. Microarray analysis of pneumococcal gene expression during invasive disease. Infect Immun **72:**5582-5596.
273. **Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard.** 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. J Exp Med **146:**257-270.
274. **Panina, E. M., A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci U S A **100:**9912-9917.
275. **Parker, D., G. Soong, P. Planet, J. Brower, A. J. Ratner, and A. Prince.** 2009. The NanA neuraminidase of Streptococcus pneumoniae is involved in biofilm formation. Infect Immun **77:**3722-3730.
276. **Pastor, P., F. Medley, and T. V. Murphy.** 1998. Invasive pneumococcal disease in Dallas County, Texas: results from population-based surveillance in 1995. Clin Infect Dis **26:**590-595.
277. **Paterson, G. K., and T. J. Mitchell.** 2006. Innate immunity and the pneumococcus. Microbiology **152:**285-293.
278. **Paton, J. C.** 1998. Novel pneumococcal surface proteins: role in virulence and vaccine potential. Trends Microbiol **6:**85-87; discussion 87-88.
279. **Paton, J. C., B. Rowan-Kelly, and A. Ferrante.** 1984. Activation of human complement by the pneumococcal toxin pneumolysin. Infect Immun **43:**1085-1087.
280. **Peltola, H.** 2001. Burden of meningitis and other severe bacterial infections of children in africa: implications for prevention. Clin Infect Dis **32:**64-75.
281. **Pelton, S. I., A. M. Loughlin, and C. D. Marchant.** 2004. Seven valent pneumococcal conjugate vaccine immunization in two Boston communities: changes in serotypes and antimicrobial susceptibility among Streptococcus pneumoniae isolates. Pediatr Infect Dis J **23:**1015-1022.
282. **Pepys, M. B., and G. M. Hirschfield.** 2003. C-reactive protein: a critical update. J Clin Invest **111:**1805-1812.
283. **Plotkowski, M. C., E. Puchelle, G. Beck, J. Jacquot, and C. Hannoun.** 1986. Adherence of type I Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus. Am Rev Respir Dis **134:**1040-1044.
284. **Porat, N., G. Barkai, M. R. Jacobs, R. Trefler, and R. Dagan.** 2004. Four Antibiotic-Resistant Streptococcus pneumoniae Clones Unrelated to the Pneumococcal Conjugate Vaccine Serotypes, Including 2 New Serotypes, Causing Acute Otitis Media in Southern Israel. J Infect Dis **189:**385-392.
285. **Poulsen, K., J. Reinholdt, and M. Kilian.** 1996. Characterization of the Streptococcus pneumoniae immunoglobulin A1 protease gene (iga) and its translation product. Infect Immun **64:**3957-3966.

286. **Prymula, R., P. Peeters, V. Chrobok, P. Kriz, E. Novakova, E. Kaliskova, I. Kohl, P. Lommel, J. Poolman, J. P. Prieels, and L. Schuerman.** 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet **367:**740-748.
287. **Quataert, S. A., C. S. Kirch, L. J. Wiedl, D. C. Phipps, S. Strohmeyer, C. O. Cimino, J. Skuse, and D. V. Madore.** 1995. Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin Diagn Lab Immunol **2:**590-597.
288. **Quataert, S. A., K. Rittenhouse-Olson, C. S. Kirch, B. Hu, S. Secor, N. Strong, and D. V. Madore.** 2004. Assignment of weight-based antibody units for 13 serotypes to a human antipneumococcal standard reference serum, lot 89-S(f). Clin Diagn Lab Immunol **11:**1064-1069.
289. **Quin, L. R., S. Carmicle, S. Dave, M. K. Pangburn, J. P. Evenhuis, and L. S. McDaniel.** 2005. In vivo binding of complement regulator factor H by Streptococcus pneumoniae. J Infect Dis **192:**1996-2003.
290. **Quin, L. R., Q. C. Moore, 3rd, and L. S. McDaniel.** 2007. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infect Immun **75:**2067-2070.
291. **Quin, L. R., C. Onwubiko, S. Carmicle, and L. S. McDaniel.** 2006. Interaction of clinical isolates of Streptococcus pneumoniae with human complement factor H. FEMS Microbiol Lett **264:**98-103.
292. **Quin, L. R., C. Onwubiko, Q. C. Moore, M. F. Mills, L. S. McDaniel, and S. Carmicle.** 2007. Factor H binding to PspC of Streptococcus pneumoniae increases adherence to human cell lines in vitro and enhances invasion of mouse lungs in vivo. Infect Immun **75:**4082-4087.
293. **Rapola, S., V. Jantti, R. Haikala, R. Syrjanen, G. M. Carlone, J. S. Sampson, D. E. Briles, J. C. Paton, A. K. Takala, T. M. Kilpi, and H. Kayhty.** 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. J Infect Dis **182:**1146-1152.
294. **Redd, S. C., G. W. Rutherford, and M. A. Sande.** 1990. The role of human immunodeficiency virus infection in pneumococcal bacteremia in San Francisco residents. Journal of infectious diseases **162:**1012-1017.
295. **Reinert, R., M. R. Jacobs, and S. L. Kaplan.** 2010. Pneumococcal disease caused by serotype 19A: review of the literature and implications for future vaccine development. Vaccine **28:**4249-4259.
296. **Ren, B., M. A. McCrory, C. Pass, D. C. Bullard, C. M. Ballantyne, Y. Xu, D. E. Briles, and A. J. Szalai.** 2004. The virulence function of Streptococcus pneumoniae surface protein A involves inhibition of complement activation and

impairment of complement receptor-mediated protection. J Immunol **173:**7506-7512.

297. **Ren, B., A. J. Szalai, S. K. Hollingshead, and D. E. Briles.** 2004. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun **72:**114-122.
298. **Ren, B., A. J. Szalai, O. Thomas, S. K. Hollingshead, and D. E. Briles.** 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. Infect Immun **71:**75-85.
299. **Riboldi-Tunnicliffe, A., N. W. Isaacs, and T. J. Mitchell.** 2005. 1.2 Angstroms crystal structure of the S. pneumoniae PhtA histidine triad domain a novel zinc binding fold. FEBS Lett **579:**5353-5360.
300. **Richards, L., D. M. Ferreira, E. N. Miyaji, P. W. Andrew, and A. Kadioglu.** 2010. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology **215:**251-263.
301. **Ring, A., J. N. Weiser, and E. I. Tuomanen.** 1998. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. J Clin Invest **102:**347-360.
302. **Rioux, S., C. Neyt, E. Di Paolo, L. Turpin, N. Charland, S. Labbe, M. C. Mortier, T. J. Mitchell, C. Feron, D. Martin, and J. T. Poolman.** 2010. Transcriptional regulation, occurrence and putative role of the Pht Family of Streptococcus pneumoniae. Microbiology.
303. **Robbins, J. B., R. Austrian, C. J. Lee, S. C. Rastogi, G. Schiffman, J. Henrichsen, P. H. Makela, C. V. Broome, R. R. Facklam, R. H. Tiesjema, and et al.** 1983. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. J Infect Dis **148:**1136-1159.
304. **Robinson, D. A., K. M. Edwards, K. B. Waites, D. E. Briles, M. J. Crain, and S. K. Hollingshead.** 2001. Clones of Streptococcus pneumoniae isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. J Infect Dis **183:**1501-1507.
305. **Robson, R. L., N. A. Reed, and R. T. Horvat.** 2006. Differential activation of inflammatory pathways in A549 type II pneumocytes by Streptococcus pneumoniae strains with different adherence properties. BMC Infect Dis **6:**71.
306. **Roche, H., B. Ren, L. S. McDaniel, A. Hakansson, and D. E. Briles.** 2003. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of Streptococcus pneumoniae. Infect Immun **71:**4498-4505.
307. **Romanello, V., M. Marcacci, F. Dal Molin, M. Moschioni, S. Censini, A. Covacci, A. G. Baritussio, C. Montecucco, and F. Tonello.** 2006. Cloning,

expression, purification, and characterization of Streptococcus pneumoniae IgA1 protease. Protein Expr Purif **45:**142-149.

308. **Romero-Steiner, S., D. Libutti, L. B. Pais, J. Dykes, P. Anderson, J. C. Whitin, H. L. Keyserling, and G. M. Carlone.** 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against Streptococcus pneumoniae using differentiated HL-60 cells. Clin Diagn Lab Immunol **4:**415-422.
309. **Romero-Steiner, S., T. Pilishvili, J. S. Sampson, S. E. Johnson, A. Stinson, G. M. Carlone, and E. W. Ades.** 2003. Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. Clin Diagn Lab Immunol **10:**246-251.
310. **Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R. Masure.** 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. Mol Microbiol **25:**819-829.
311. **Ross, G. D.** 1989. Complement and complement receptors. Curr Opin Immunol **2:**50-62.
312. **Rothlein, R., and T. A. Springer.** 1985. Complement receptor type three-dependent degradation of opsonized erythrocytes by mouse macrophages. J Immunol **135:**2668-2672.
313. **Roumenina, L. T., M. M. Ruseva, A. Zlatarova, R. Ghai, M. Kolev, N. Olova, M. Gadjeva, A. Agrawal, B. Bottazzi, A. Mantovani, K. B. Reid, U. Kishore, and M. S. Kojouharova.** 2006. Interaction of C1q with IgG1, C-reactive protein and pentraxin 3: mutational studies using recombinant globular head modules of human C1q A, B, and C chains. Biochemistry **45:**4093-4104.
314. **Roy, S., K. Knox, S. Segal, D. Griffiths, C. E. Moore, K. I. Welsh, A. Smarason, N. P. Day, W. L. McPheat, D. W. Crook, and A. V. Hill.** 2002. MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet **359:**1569-1573.
315. **Rubins, J. B., D. Charboneau, C. Fasching, A. M. Berry, J. C. Paton, J. E. Alexander, P. W. Andrew, T. J. Mitchell, and E. N. Janoff.** 1996. Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. Am J Respir Crit Care Med **153:**1339-1346.
316. **Rubins, J. B., P. G. Duane, D. Clawson, D. Charboneau, J. Young, and D. E. Niewoehner.** 1993. Toxicity of pneumolysin to pulmonary alveolar epithelial cells. Infect Immun **61:**1352-1358.
317. **Rubins, J. B., and E. N. Janoff.** 1998. Pneumolysin: a multifunctional pneumococcal virulence factor. J Lab Clin Med **131:**21-27.
318. **Rubins, J. B., A. H. Paddock, D. Charboneau, A. M. Berry, J. C. Paton, and E. N. Janoff.** 1998. Pneumolysin in pneumococcal adherence and colonization. Microb Pathog **25:**337-342.

319. **Rus, H., C. Cudrici, and F. Niculescu.** 2005. The role of the complement system in innate immunity. Immunol Res **33:**103-112.
320. **Sa-Leao, R., F. Pinto, S. Aguiar, S. Nunes, J. A. Carrico, N. Frazao, N. Goncalves-Sousa, J. Melo-Cristino, H. de Lencastre, and M. Ramirez.** 2011. Invasiveness of pneumococcal serotypes and clones circulating in Portugal before widespread use of conjugate vaccines revealing heterogeneous behavior of clones expressing the same serotype. J Clin Microbiol.
321. **Saaka, M., B. J. Okoko, R. C. Kohberger, S. Jaffar, G. Enwere, E. E. Biney, C. Oluwalana, A. Vaughan, S. M. Zaman, L. Asthon, D. Goldblatt, B. M. Greenwood, F. T. Cutts, and R. A. Adegbola.** 2008. Immunogenicity and serotype-specific efficacy of a 9-valent pneumococcal conjugate vaccine (PCV-9) determined during an efficacy trial in The Gambia. Vaccine **26:**3719-3726.
322. **Sabharwal, V., S. Ram, M. Figueira, I. H. Park, and S. I. Pelton.** 2009. Role of complement in host defense against pneumococcal otitis media. Infect Immun **77:**1121-1127.
323. **Saeland, E., J. H. Leusen, G. Vidarsson, W. Kuis, E. A. Sanders, I. Jonsdottir, and J. G. van de Winkel.** 2003. Role of leukocyte immunoglobuin G receptors in vaccine-induced immunity to Streptococcus pneumoniae. J Infect Dis **187:**1686-1693.
324. **Sahu, A., and M. K. Pangburn.** 1995. Tyrosine is a potential site for covalent attachment of activated complement component C3. Mol Immunol **32:**711-716.
325. **Sampson, J. S., Z. Furlow, A. M. Whitney, D. Williams, R. Facklam, and G. M. Carlone.** 1997. Limited diversity of Streptococcus pneumoniae psaA among pneumococcal vaccine serotypes. Infect Immun **65:**1967-1971.
326. **Sandgren, A., B. Albiger, C. J. Orihuela, E. Tuomanen, S. Normark, and B. Henriques-Normark.** 2005. Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans. J Infect Dis **192:**791-800.
327. **Sandgren, A., K. Sjostrom, B. Olsson-Liljequist, B. Christensson, A. Samuelsson, G. Kronvall, and B. Henriques Normark.** 2004. Effect of Clonal and Serotype-Specific Properties on the Invasive Capacity of Streptococcus pneumoniae. J Infect Dis **189:**785-796.
328. **Schuchat, A., T. Hilger, E. Zell, M. M. Farley, A. Reingold, L. Harrison, L. Lefkowitz, R. Danila, K. Stefonek, N. Barrett, D. Morse, and R. Pinner.** 2001. Active bacterial core surveillance of the emerging infections program network. Emerg Infect Dis **7:**92-99.
329. **Schuerman, L., R. Prymula, I. Henckaerts, and J. Poolman.** 2007. ELISA IgG concentrations and opsonophagocytic activity following pneumococcal protein D conjugate vaccination and relationship to efficacy against acute otitis media. Vaccine **25:**1962-1968.
330. **Shaper, M., S. K. Hollingshead, W. H. Benjamin, Jr., and D. E. Briles.** 2004. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and

antibody to PspA enhances killing of Pneumococci by apolactoferrin. Infect Immun **72:**5031-5040.

331. **Shapiro, E. D., A. T. Berg, R. Austrian, D. Schroeder, V. Parcells, A. Margolis, R. K. Adair, and J. D. Clemens.** 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N Engl J Med **325:**1453-1460.
332. **Shouval, D. S., D. Greenberg, N. Givon-Lavi, N. Porat, and R. Dagan.** 2006. Site-specific disease potential of individual Streptococcus pneumoniae serotypes in pediatric invasive disease, acute otitis media and acute conjunctivitis. Pediatr Infect Dis J **25:**602-607.
333. **Siber, G. R., K. P. Klugman, and P. H. Mäkelä.** 2008. Pneumococcal vaccines, chapter 7 by Hostetter M. ASM Press, Washington, DC USA.
334. **Sim, R. B., T. M. Twose, D. S. Paterson, and E. Sim.** 1981. The covalent-binding reaction of complement component C3. Biochem J **193:**115-127.
335. **Simell, B., P. Ahokas, M. Lahdenkari, J. Poolman, I. Henckaerts, T. M. Kilpi, and H. Kayhty.** 2009. Pneumococcal carriage and acute otitis media induce serum antibodies to pneumococcal surface proteins CbpA and PhtD in children. Vaccine **27:**4615-4621.
336. **Simell, B., T. M. Kilpi, and H. Kayhty.** 2002. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children. J Infect Dis **186:**1106-1114.
337. **Simell, B., M. Korkeila, H. Pursiainen, T. M. Kilpi, and H. Kayhty.** 2001. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. J Infect Dis **183:**887-896.
338. **Simell, B., M. Lahdenkari, A. Reunanen, H. Kayhty, and M. Vakevainen.** 2008. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular polysaccharides and virulence-associated proteins. Clin Vaccine Immunol **15:**1391-1397.
339. **Simell, B., M. Melin, M. Lahdenkari, D. E. Briles, S. K. Hollingshead, T. M. Kilpi, and H. Kayhty.** 2007. Antibodies to pneumococcal surface protein A families 1 and 2 in serum and saliva of children and the risk of pneumococcal acute otitis media. J Infect Dis **196:**1528-1536.
340. **Sjoberg, A. P., L. A. Trouw, F. D. McGrath, C. E. Hack, and A. M. Blom.** 2006. Regulation of complement activation by C-reactive protein: targeting of the inhibitory activity of C4b-binding protein. J Immunol **176:**7612-7620.
341. **Sjostrom, K., C. Spindler, A. Ortqvist, M. Kalin, A. Sandgren, S. Kuhlmann-Berenzon, and B. Henriques-Normark.** 2006. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. Clin Infect Dis **42:**451-459.
342. **Sleeman, K. L., D. Griffiths, F. Shackley, L. Diggle, S. Gupta, M. C. Maiden, E. R. Moxon, D. W. Crook, and T. E. Peto.** 2006. Capsular serotype-specific

attack rates and duration of carriage of Streptococcus pneumoniae in a population of children. J Infect Dis **194:**682-688.

343. **Smillie, W. G., and F. S. Leeder.** 1934. Epidemiology of Lobar Pneumonia. Am J Public Health Nations Health **24:**129-138.
344. **Smith, B. L., and M. K. Hostetter.** 2000. C3 as substrate for adhesion of Streptococcus pneumoniae. J Infect Dis **182:**497-508.
345. **Soininen, A., M. Karpala, S. L. Wahlman, H. Lehtonen, and H. Kayhty.** 2002. Specificities and opsonophagocytic activities of antibodies to pneumococcal capsular polysaccharides in sera of unimmunized young children. Clin Diagn Lab Immunol **9:**1032-1038.
346. **Soininen, A., H. Pursiainen, T. Kilpi, and H. Kayhty.** 2001. Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association with pneumococcal carriage and acute otitis media in young children. J Infect Dis **184:**569-576.
347. **Soininen, A., I. Seppala, T. Nieminen, J. Eskola, and H. Kayhty.** 1999. IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. Vaccine **17:**1889-1897.
348. **Sombrero, L., A. Nissinen, G. Esparar, M. Lindgren, L. Siira, and A. Virolainen.** 2008. Low incidence of antibiotic resistance among invasive and nasopharyngeal isolates of Streptococcus pneumoniae from children in rural Philippines between 1994 and 2000. Eur J Clin Microbiol Infect Dis.
349. **Sorensen, U. B., J. Henrichsen, H. C. Chen, and S. C. Szu.** 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of Streptococcus pneumoniae revealed by immunochemical methods. Microb Pathog **8:**325-334.
350. **Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, N. Schnitzler, R. Lutticken, and A. Podbielski.** 1999. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of Streptococcus agalactiae to human laminin. Infect Immun **67:**871-878.
351. **Stillman, E. G.** 1917. Further studies on the epidemiology of lobar pneumonia. J Exp Med **26:**513-535.
352. **Strunk, R. C., D. M. Eidlen, and R. J. Mason.** 1988. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. J Clin Invest **81:**1419-1426.
353. **Syrjänen, R. K., T. M. Kilpi, T. H. Kaijalainen, E. E. Herva, and A. K. Takala.** 2001. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. J Infect Dis **184:**451-459.
354. **Tack, B. F., R. A. Harrison, J. Janatova, M. L. Thomas, and J. W. Prahl.** 1980. Evidence for presence of an internal thiolester bond in third component of human complement. Proc Natl Acad Sci U S A **77:**5764-5768.

355. **Teele, D. W., J. O. Klein, and B. Rosner.** 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J Infect Dis **160:**83-94.
356. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. Science **293:**498-506.
357. **Thiel, S., T. Vorup-Jensen, C. M. Stover, W. Schwaeble, S. B. Laursen, K. Poulsen, A. C. Willis, P. Eggleton, S. Hansen, U. Holmskov, K. B. Reid, and J. C. Jensenius.** 1997. A second serine protease associated with mannan-binding lectin that activates complement. Nature **386:**506-510.
358. **Toikka, P., S. Nikkari, O. Ruuskanen, M. Leinonen, and J. Mertsola.** 1999. Pneumolysin PCR-based diagnosis of invasive pneumococcal infection in children. J Clin Microbiol **37:**633-637.
359. **Trzcinski, K., C. Thompson, R. Malley, and M. Lipsitch.** 2005. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. Infect Immun **73:**7043-7046.
360. **Trzcinski, K., C. M. Thompson, and M. Lipsitch.** 2003. Construction of Otherwise Isogenic Serotype 6B, 7F, 14, and 19F Capsular Variants of Streptococcus pneumoniae Strain TIGR4. Appl Environ Microbiol **69:**7364-7370.
361. **Trzcinski, K., C. M. Thompson, A. Srivastava, A. Basset, R. Malley, and M. Lipsitch.** 2008. Protection against nasopharyngeal colonization by Streptococcus pneumoniae is mediated by antigen-specific CD4+ T cells. Infect Immun **76:**2678-2684.
362. **Tschopp, J., A. Engel, and E. R. Podack.** 1984. Molecular weight of poly(C9). 12 to 18 C9 molecules form the transmembrane channel of complement. J Biol Chem **259:**1922-1928.
363. **Tschopp, J., H. J. Muller-Eberhard, and E. R. Podack.** 1982. Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9. Nature **298:**534-538.
364. **Tseng, H. J., A. G. McEwan, J. C. Paton, and M. P. Jennings.** 2002. Virulence of Streptococcus pneumoniae: PsaA mutants are hypersensitive to oxidative stress. Infect Immun **70:**1635-1639.

365. **Tu, A. H., R. L. Fulgham, M. A. McCrory, D. E. Briles, and A. J. Szalai.** 1999. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun **67:**4720-4724.
366. **Waite, R. D., J. K. Struthers, and C. G. Dowson.** 2001. Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol Microbiol **42:**1223-1232.
367. **Valles, X., A. Roca, F. Lozano, L. Morais, B. Suarez, F. Casals, I. Mandomando, B. Sigauque, D. Nhalungo, C. Esquinas, L. Quinto, P. L. Alonso, and A. Torres.** 2010. Serotype-specific pneumococcal disease may be influenced by mannose-binding lectin deficiency. Eur Respir J **36:**856-863.
368. **Wallis, R., D. A. Mitchell, R. Schmid, W. J. Schwaeble, and A. H. Keeble.** 2010. Paths reunited: Initiation of the classical and lectin pathways of complement activation. Immunobiology **215:**1-11.
369. **van de Beek, D., B. Schmand, J. de Gans, M. Weisfelt, H. Vaessen, J. Dankert, and M. Vermeulen.** 2002. Cognitive impairment in adults with good recovery after bacterial meningitis. J Infect Dis **186:**1047-1052.
370. **van Rossum, A. M., E. S. Lysenko, and J. N. Weiser.** 2005. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. Infect Immun **73:**7718-7726.
371. **Ward, H. K., and J. F. Enders.** 1933. An Analysis of the Opsonic and Tropic Action of Normal and Immune Sera Basedon Experiments with the Pneumococcus. J Exp Med **57:**527-547.
372. **Wartha, F., K. Beiter, B. Albiger, J. Fernebro, A. Zychlinsky, S. Normark, and B. Henriques-Normark.** 2007. Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol **9:**1162-1171.
373. **Watford, W. T., A. J. Ghio, and J. R. Wright.** 2000. Complement-mediated host defense in the lung. Am J Physiol Lung Cell Mol Physiol **279:**L790-798.
374. **Watson, D. A., and D. M. Musher.** 1990. Interruption of capsule production in Streptococcus pneumonia serotype 3 by insertion of transposon Tn916. Infect Immun **58:**3135-3138.
375. **Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon.** 1976. Control of the amplification convertase of complement by the plasma protein beta1H. Proc Natl Acad Sci U S A **73:**3268-3272.
376. **Weinberger, D. M., R. Dagan, N. Givon-Lavi, G. Regev-Yochay, R. Malley, and M. Lipsitch.** 2008. Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. J Infect Dis **197:**1511-1518.
377. **Weinberger, D. M., Z. B. Harboe, E. A. Sanders, M. Ndiritu, K. P. Klugman, S. Ruckinger, R. Dagan, R. Adegbola, F. Cutts, H. L. Johnson, K. L. O'Brien, J. Anthony Scott, and M. Lipsitch.** 2010. Association of serotype with risk of

death due to pneumococcal pneumonia: a meta-analysis. Clin Infect Dis **51:**692-699.

378. **Weinberger, D. M., K. Trzcinski, Y. J. Lu, D. Bogaert, A. Brandes, J. Galagan, P. W. Anderson, R. Malley, and M. Lipsitch.** 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog **5:**e1000476.
379. **Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure.** 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun **62:**2582-2589.
380. **Weiser, J. N., D. Bae, H. Epino, S. B. Gordon, M. Kapoor, L. A. Zenewicz, and M. Shchepetov.** 2001. Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of Streptococcus pneumoniae. Infect Immun **69:**5430-5439.
381. **Weiser, J. N., D. Bae, C. Fasching, R. W. Scamurra, A. J. Ratner, and E. N. Janoff.** 2003. Antibody-enhanced pneumococcal adherence requires IgA1 protease. Proc Natl Acad Sci U S A **100:**4215-4220.
382. **Weiser, J. N., Z. Markiewicz, E. I. Tuomanen, and J. H. Wani.** 1996. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by Streptococcus pneumoniae. Infect Immun **64:**2240-2245.
383. **Vesikari, T., J. Wysocki, B. Chevallier, A. Karvonen, H. Czajka, J. P. Arsene, P. Lommel, I. Dieussaert, and L. Schuerman.** 2009. Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. Pediatr Infect Dis J **28:**S66-76.
384. **White, P., A. Hermansson, C. Svanborg, D. Briles, and K. Prellner.** 1999. Effects of active immunization with a pneumococcal surface protein (PspA) and of locally applied antibodies in experimental otitis media. ORL J Otorhinolaryngol Relat Spec **61:**206-211.
385. **Whitney, C. G., M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, R. Lynfield, A. Reingold, P. R. Cieslak, T. Pilishvili, D. Jackson, R. R. Facklam, J. H. Jorgensen, and A. Schuchat.** 2003. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med **348:**1737-1746.
386. **Whitney, C. G., M. M. Farley, J. Hadler, L. H. Harrison, C. Lexau, A. Reingold, L. Lefkowitz, P. R. Cieslak, M. Cetron, E. R. Zell, J. H. Jorgensen, and A. Schuchat.** 2000. Increasing prevalence of multidrug-resistant Streptococcus pneumoniae in the United States. N Engl J Med **343:**1917-1924.
387. **Whitney, C. G., T. Pilishvili, M. M. Farley, W. Schaffner, A. S. Craig, R. Lynfield, A. C. Nyquist, K. A. Gershman, M. Vazquez, N. M. Bennett, A. Reingold, A. Thomas, M. P. Glode, E. R. Zell, J. H. Jorgensen, B. Beall, and A. Schuchat.** 2006. Effectiveness of seven-valent pneumococcal conjugate vaccine

against invasive pneumococcal disease: a matched case-control study. Lancet **368:**1495-1502.

388. **Winkelstein, J. A.** 1981. The Role of Complement in the Host´s Defence Against *Streptococcus pneumoniae*. Reviews of Infectious Diseases **3:**289-298.
389. **Wizemann, T. M., J. H. Heinrichs, J. E. Adamou, A. L. Erwin, C. Kunsch, G. H. Choi, S. C. Barash, C. A. Rosen, H. R. Masure, E. Tuomanen, A. Gayle, Y. A. Brewah, W. Walsh, P. Barren, R. Lathigra, M. Hanson, S. Langermann, S. Johnson, and S. Koenig.** 2001. Use of a whole genome approach to identify vaccine molecules affording protection against Streptococcus pneumoniae infection. Infect Immun **69:**1593-1598.
390. **Volanakis, J. E., and M. H. Kaplan.** 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc Soc Exp Biol Med **136:**612-614.
391. **Woolley, A. L., K. A. Kirk, A. M. Neumann, Jr., S. M. McWilliams, J. Murray, D. Freind, and B. J. Wiatrak.** 1999. Risk factors for hearing loss from meningitis in children: the Children's Hospital experience. Arch Otolaryngol Head Neck Surg **125:**509-514.
392. **Wu, H. Y., M. H. Nahm, Y. Guo, M. W. Russell, and D. E. Briles.** 1997. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with Streptococcus pneumoniae. J Infect Dis **175:**839-846.
393. **Wuorimaa, T., R. Dagan, M. Vakevainen, F. Bailleux, R. Haikala, M. Yaich, J. Eskola, and H. Kayhty.** 2001. Avidity and subclasses of IgG after immunization of infants with an 11-valent pneumococcal conjugate vaccine with or without aluminum adjuvant. J Infect Dis **184:**1211-1215.
394. **Wuorimaa, T. K., R. Dagan, F. Bailleux, R. Haikala, N. Ekstrom, J. Eskola, M. Yaich, and H. Kayhty.** 2005. Functional activity of antibodies after immunization of Finnish and Israeli infants with an 11-valent pneumococcal conjugate vaccine. Vaccine **23:**5328-5332.
395. **Yeh, S. H., A. Gurtman, D. C. Hurley, S. L. Block, R. H. Schwartz, S. Patterson, K. U. Jansen, J. Love, W. C. Gruber, E. A. Emini, and D. A. Scott.** 2010. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine in infants and toddlers. Pediatrics **126:**e493-505.
396. **Ying, S. C., A. T. Gewurz, H. Jiang, and H. Gewurz.** 1993. Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14-26 and 76-92 of the A chain collagen-like region of C1q. J Immunol **150:**169-176.
397. **Yother, J., and D. E. Briles.** 1992. Structural properties and evolutionary relationships of PspA, a surface protein of Streptococcus pneumoniae, as revealed by sequence analysis. J Bacteriol **174:**601-609.

398. **Yother, J., G. L. Handsome, and D. E. Briles.** 1992. Truncated forms of PspA that are secreted from Streptococcus pneumoniae and their use in functional studies and cloning of the pspA gene. J Bacteriol **174:**610-618.
399. **Yother, J., and J. M. White.** 1994. Novel surface attachment mechanism of the Streptococcus pneumoniae protein PspA. J Bacteriol **176:**2976-2985.
400. **Yuste, J., M. Botto, S. E. Bottoms, and J. S. Brown.** 2007. Serum amyloid P aids complement-mediated immunity to Streptococcus pneumoniae. PLoS Pathog **3:**1208-1219.
401. **Yuste, J., M. Botto, J. C. Paton, D. W. Holden, and J. S. Brown.** 2005. Additive inhibition of complement deposition by pneumolysin and PspA facilitates Streptococcus pneumoniae septicemia. J Immunol **175:**1813-1819.
402. **Yuste, J., S. Khandavilli, N. Ansari, K. Muttardi, L. Ismail, C. Hyams, J. Weiser, T. Mitchell, and J. S. Brown.** 2010. The effects of PspC on complement-mediated immunity to Streptococcus pneumoniae vary with strain background and capsular serotype. Infect Immun **78:**283-292.
403. **Yuste, J., S. Khandavilli, N. Ansari, K. Muttardi, L. Ismail, C. Hyams, J. Weiser, T. Mitchell, and J. S. Brown.** 2009. The effects of PspC on complement-mediated immunity to Streptococcus pneumoniae vary with strain background and capsular serotype. Infect Immun.
404. **Yuste, J., A. Sen, L. Truedsson, G. Jonsson, L. S. Tay, C. Hyams, H. E. Baxendale, F. Goldblatt, M. Botto, and J. S. Brown.** 2008. Impaired opsonization with C3b and phagocytosis of Streptococcus pneumoniae in sera from subjects with defects in the classical complement pathway. Infect Immun **76:**3761-3770.
405. **Zhang, J. R., K. E. Mostov, M. E. Lamm, M. Nanno, S. Shimida, M. Ohwaki, and E. Tuomanen.** 2000. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. Cell **102:**827-837.
406. **Zhang, Y., A. W. Masi, V. Barniak, K. Mountzouros, M. K. Hostetter, and B. A. Green.** 2001. Recombinant PhpA protein, a unique histidine motif-containing protein from Streptococcus pneumoniae, protects mice against intranasal pneumococcal challenge. Infect Immun **69:**3827-3836.
407. **Zhang, Z., T. B. Clarke, and J. N. Weiser.** 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest **119:**1899-1909.
408. **Ziccardi, R. J., B. Dahlback, and H. J. Muller-Eberhard.** 1984. Characterization of the interaction of human C4b-binding protein with physiological ligands. J Biol Chem **259:**13674-13679.